Selection and analysis of predictive fed-state gastric biorelevant media

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Selection and analysis of predictive fed-state gastric biorelevant media

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Declaration of any previous submission of the work

The material presented here for examination for the award of a higher degree by research has not been incorporated into a submission for another degree.

Candidate's signature 

Declaration of authorship

I am the author of this thesis, and the work described therein was carried out by myself personally.

Candidate's signature 

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List of Abbreviations

ACN: Acetonitrile

$AUC_{0\rightarrow\infty}$: Area under the curve from time 0 extrapolated to infinite time

$AUC_{0\rightarrow\infty}$: Area under the curve from time 0 extrapolated to time t

API: Active pharmaceutical ingredient

ATE: Atenolol

ATORV: Atorvastatin calcium

ATOV: Atovaquone

Aq sol $_{pH\ 5}$ (in Chapter 4): theoretical aqueous solubility at pH 5 in μg/mL

AZITH: Azithromycin

BA/BE: Bioavailability/bioequivalence

BCS: Biopharmaceutics classification system

C_{max}: Maximum plasma concentration

$C_{part\ max}$: Maximum drug percentage partitioned to fat

$C_{part\ t}$: Drug percentage partitioned to the fat at time t

CEL: Celecoxib

DAN: Danazol

DModY: Distance to model; residuals of Y

EMA: European Medicines Agency

EtOH: Ethanol

FDA: Food and Drug Administration

FeSSGF: Fed State Simulated Gastric Fluid

FeSSGFEm: Fed State Simulated Gastric Fluid prepared using a lipid emulsion,

$FeSSGF_{nm}$: Non-milk-based Fed State Simulated Gastric Fluid

$FeSSGF_{hf}$: Fed State Simulated Gastric Fluid prepared with high fat milk (5% w/v)

$FeSSGF_{sk}$: Fed State Simulated Gastric Fluid prepared with skimmed milk

FUR: Furosemide

GF/D: Glass microfiber

GI: Gastrointestinal

HGL: Human gastric lipase

HLB: Hydrophilic-lipophilic balance

ITR: Itraconazole

IVIVC: *in vitro-in vivo* correlation

KET: Ketoconazole

LAP: Lapatinib

Log aq sol (in Chapter 2): Logarithm of drug aqueous solubility in mg/mL

Log $D_{pH\ 5}$: octanol/water distribution coefficient at pH 5

Log P: octanol/water partition coefficient

LOQ: Limit of quantification

MeOH: Methanol

mEq/L/ΔpH: Milliequivalent per litter per pH unit difference

METF: Metformin hydrochloride

METOP: Metoprolol tartrate

MFGM: Milk fat globule membrane

MLR: Multiple linear regression

mN/m: Millinewton per meter

mOsm/kg: Milliosmolar/kilogram

MW: Molecular weight

MWCO: Molecular weight cut off

NaCas: Sodium caseinate

NIF: Nifedipine

PAR: Paracetamol

PC: Phosphatidylcholine

PE: Phosphatidylethanolamine

PLS: Partial least squares

PP: Protein precipitation

PRAV: Pravastatin sodium

PRESS: Predicted residual error sum of squares

PROP: Propafenone hydrochloride

Prot b fr: Drug protein bound fraction in serum proteins

Q^2: Crossvalidated coefficient of determination

R^2: Coefficient of determination

RC: Regenerated cellulose

R_e: Raynold's number

RN: Rhizopus niveus

Rpm: Rounds per minute

SDS (or SLS): Sodium dodecyl sulphate

SGF: Simulated gastric fluid

SPE: Solid phase extraction

Spm: Shakes per minute

TCA: Trichloroacetic acid

TFA: Trifluoroacetic acid

T_{max}: Time to reach maximum plasma concentration

VIF: Variance inflation factor

Union fr: Drug unionised fraction at pH = 5

USP: United States Pharmacopoeia

Abstract

Dissolution testing is a tool towards prediction of dosage form behaviour under physiologically relevant conditions. The use of simple aqueous media dictated by the pharmacopoeias cannot predict the drug's *in vivo* response as their physicochemical properties differ significantly from the complex environment of the gastrointestinal tract. To improve the predictive potential of drug dissolution, the development of more "biorelevant" media is essential. In particular, simulating drug dissolution in the gastric environment after administration of a meal still remains a challenge. Furthermore, except for the optimisation of the medium composition, the analysis of these usually complex heterogeneous media has also been challenging, due to the lack of a unified guideline for the selection of medium and analytical assay. The principal aim of the thesis was the development of a simple and robust analytical methodology, optimised on the basis of the drug's physicochemical properties, interaction with excipients in a formulation and fat partitioning behaviour. The potential use of sample clean-up techniques including protein precipitation (PP) and solid phase extraction (SPE) was investigated. Optimised clean-up protocols were successfully used for extraction and quantification of drugs of a wide range of lipophilicity in milk-based fed biorelevant media. It was demonstrated that prior knowledge of the active ingredient's physicochemical properties, such as log P, aqueous solubility, ionisation and protein binding can be used towards the selection of optimum extraction conditions. Moreover, the presence of certain excipients, when mixed with the APIs can significantly affect the methods' efficiency and must therefore be taken into consideration during analytical method development. Interactions between active ingredients and lipid part of the fed gastric content were also investigated, via development of biphasic "drug partition to fat" *in vitro* setups. The rate of drug partition to fat was successfully predicted based on the drug's physicochemical properties and *in vivo* food effect. Finally, a simpler medium, alternative to Fed State Simulated Gastric Fluid (FeSSGF) was developed, requiring a less laborious extraction protocol. Overall, this thesis has provided useful insight on the critical aspects of fed gastric medium and analytical methodology development. It provides a point of reference for future work on better understanding on drug solubilisation in the gastric fed state and correlation with *in vivo* food effect.

Aims and Objectives

The experimental work of the thesis is divided in three main parts. The first part will assess critical aspects in the analysis of APIs in biorelevant milk-based fed gastric media, in the presence and absence of excipients, with an intend to provide a unified guideline, applicable for wide range of drugs of different physicochemical properties. The second part will focus on the interactions between the model compounds and the lipid portion of the biorelevant media developed *in vitro*, and will try to correlate the partition and release to and from fat with drug food effect observed *in vivo* with and with drug's physicochemical characteristics. Finally, the last part will propose an alternative medium of similar physicochemical properties with the widely used FeSSGF, as a first step towards the development of a gastric fed medium requiring less laborious sample clean-up techniques prior to drug analysis.

The overall aim of the thesis is to design, develop and validate a universal analytical method for the quantification of drugs in fed state gastric media including sample clean-up, extraction and evaluation of drug distribution in the aqueous and lipid parts of the medium, when the gastric environment affects the drug's behaviour. More specifically the objectives of each chapter of the current study are:

For Chapter 1: The description of the information available on standard meals used in *in vivo* food effect studies and the biorelevant media developed *in vitro* for the simulation of the gastric fed state. The study aims to give an insight on their *in vitro* characterization and understanding of the parameters affecting the drug and formulation's pharmacokinetic behaviour. The analytical techniques of the above media and the challenges in overcoming the time and complexity of such techniques are being discussed, setting the background and experimental challenges of the PhD project.

For Chapter 2: The development of a roadmap, which will serve as a guide for the analysis of APIs in milk-based fed state gastric media. More specifically, the study aims to provide a guideline for sample cleanup of drugs of a wide range of lipophilicity (from extremely hydrophilic to extremely lipophilic) and evaluate use of drug physicochemical characteristics as critical selection variables.

For Chapter 3: Evaluation of the effect of commonly used excipients in suitability of the sample treatment, as developed in the extraction of APIs in fed state media. The objective of the study is to highlight the importance of the excipient effect when analysis in performed

in drug-excipient mixtures and evaluate possible changes in the effect of drug physicochemical properties compared to the ones determined in Chapter 2, and integrate the modifications in the API extraction guideline.

For Chapter 4: Assessment of the interactions developed between drug and lipid part of the fed medium. More specifically, the aim of this part of the study is to elucidate the rate of drug partition to the lipid portion of the gastric content in the fed state, and correlate this type of behaviour with drug's physicochemical properties and/or food effect observed when administered with food in human *in vivo* studies. The study aims to develop a discriminating *in vitro* test able to determine the differences in partition rates to fat of drugs of different physicochemical characteristics which could potentially be used towards the prediction of drug food effect *in vivo*.

For Chapter 5: Development and physicochemical characterization of a novel fed gastric medium, simulating FeSSGF, which would require a less laborious extraction technique. The aim of the study is to develop and fully characterize the medium and also mimic the main drug solubilisation mechanisms of FeSSGF in drugs of a range of lipophilicity. The long-term aim of the study is to set the bases towards the development of a simpler fed gastric medium, understanding the way critical parameters affect drug solubilisation.

Chapter 1: Fed-state gastric media and drug analysis techniques: Current status and points to consider

Abstract

Gastric fed state conditions can have a significant effect on drug dissolution and absorption. *In vitro* dissolution tests with simple aqueous media cannot usually predict drugs' *in vivo* response, as several factors such as the meal content, the gastric emptying and possible interactions between food and drug formulations can affect drug's pharmacokinetics. Good understanding of the effect of the *in vivo* fed gastric conditions on the drug is essential for the development of biorelevant dissolution media simulating the gastric environment after the administration of the standard high fat meal proposed by the FDA and the EMA in bioavailability/bioequivalence (BA/BE) studies. The analysis of drugs in fed state media can be quite challenging as most analytical protocols currently employed are time consuming and labour intensive. In this review, an overview of the *in vivo* gastric conditions and the biorelevant media used for their *in vitro* simulation are described. Furthermore, an analysis of the physicochemical properties of the drugs and the formulations related to food effect is given. In terms of drug analysis, the protocols currently used for the fed state media sample treatment and analysis and the analytical challenges and needs emerging for more efficient and time saving techniques for a broad spectrum of compounds are being discussed.

Keywords: Fed state, Gastric, Biorelevant media, Drug analysis, Dissolution, Bioavailability

1.1. Introduction

In vitro dissolution studies are an integral part of quality control and drug development processes. During drug development, they are used as a tool for the selection of the appropriate excipients and the most suitable formulation type [1] and also as an *in vitro* surrogate for *in vivo* performance [2]. In quality control, they are used to ensure the batch-to-batch consistency [3–5]. Dissolution tests, as dictated by the Pharmacopoeias, cannot always provide information about the *in vivo* behaviour of the drugs, even though there are cases in which these tests can provide good *in vitro–in vivo* correlations (IVIVC). The dissolution media described in Pharmacopoeia monographs are mainly used for quality control purposes, and are not often able to predict the *in vivo* behaviour of poorly soluble drugs for which the fat content and the bile salt concentration in the gastrointestinal environment will affect their solubility and dissolution rate [6, 7]. Due to the limited ability of the simple aqueous media suggested by the Pharmacopoeias to simulate the characteristics of the gastrointestinal (GI) tract, the need for media simulating the GI physiological environment in the fasted and fed states (usually called biorelevant media) arose; in these media the physicochemical properties of the GI contents (pH, osmolality, surface tension, buffer capacity) are taken into account and physiological components such as bile salts and lecithin are incorporated [2]. Use of biorelevant media during the drug development process enables the assessment of drug's biopharmaceutic characteristics and the prediction of *in vivo* performance [2, 8].

While the fasted state gastric environment has been well studied, the more complex conditions of the fed state stomach have made the prediction of food effect a challenging task. For further information on the gastric fasted gastric state properties, the reader may refer to Vertzoni et al. [4]. In summary, pH values of approximately 1.5-1.9, pepsin output of a maximum value of 0.8 mg/mL and gastric lipase concentration of 0.1 mg/mL have been reported. Average values of bile salts have been found at a range of 80 to 275 μM, while 28-51 mN/m and 200 mOsm/kg values have been reported for surface tension and osmolality respectively. Several *in vitro* biorelevant gastric media have been used for the simulation of the gastric fed state environment and as far as the sample treatment is concerned, there is no specific protocol available and sample treatment and drug analysis are developed on a case by case basis. A good understanding of the *in vivo* conditions of the fed state stomach could lead towards the development of a suitable medium being able to simulate the gastric content and ideally overcoming the extensive treatment before the analysis that is needed with the current gastric fed state media [9, 10]. The dependence of the drug food effect on the meal content, the

role of the fat content in the solubilisation of drugs, the gastric emptying rate and the interaction with certain formulations [11] as well as the binding of drugs with metal ions and meal components are some of the parameters which have rendered the *in vitro* prediction of food effect extremely complicated.

In the current review, initially we describe the available information for the characterisation of the *in vivo* gastric fed state conditions after the administration of standard meals with an aim to provide an understanding of the effect of drug's physicochemical parameters on its *in vivo* behaviour. Then, the standard meals and the gastric biorelevant media currently being used and their interaction with drugs of different physicochemical properties are presented. In the last part, the analytical techniques used *in vitro* for sample treatment and quantification of the drug along with their challenges are discussed.

1.2. *In vivo* gastric conditions in the fed state

Gastric conditions in fasted state have been characterised in terms of pH, osmolality, surface tension, buffer capacity and protein content [12–14]. In the fed state, the determination of absolute values is more complicated than in the fasted state. The food type is an additional factor on top of other parameters responsible for the interindividual variation of the above properties such as the individual's age [15] and administered medication [16]. The role of several physicochemical parameters of the contents of the fed state stomach on drug's dissolution and absorption is reviewed.

1.2.1. Gastric secretions in the fed state

The main components of the gastric juice are hydrochloric acid (HCl), pepsinogens, mucus and water; pepsinogen is the inactive form of pepsin, activated by the presence of HCl [17]. Pepsin content is higher in the fed state stomach than in the fasted state (fasted state values = 0.11-0.22 mg/mL). Samples of gastric antrum content of twenty healthy volunteers after administration of Ensure Plus®, demonstrated pepsin values within a range from 0.26 to 0.58 mg/mL in a time period from 30 to 210 min after administration of the liquid meal [13]. Gastric lipase is also present in the stomach. It is the enzyme responsible for the digestion of fat in the upper gastrointestinal tract. Its role involves the hydrolysis of exogenously administered triglycerides to di-glycerides and fatty acids [18]. Gastric lipase has been reported to account from 10 to 30% of the total hydrolysis of triglycerides contained in a meal [19, 20] with the activity of the enzyme measured at 11.4-43.9 U/mL [21]. Its total output after administration

of a liquid meal was 22.6 ± 8.1 mg (concentration 16.7 ± 0.7 μg/mL) after administration of a liquid meal in human subjects [19].

1.2.2. Bile salts in gastric contents in the fed state

Bile salts can increase the dissolution of poorly soluble drugs by decreasing the energy barrier between the drug and the medium, by increasing the active surface area, or via micellar solubilisation [22]. Bile salt concentration in the stomach is much smaller than in the small intestine, where the bile salts are released by the gall bladder, with their concentration in the intestinal environment in the fasted state demonstrating an approximate four-fold decreased value in comparison with the fed state (1-4 mM and 10-20 mM, respectively) [23, 24]. In the gastric fed state, (after administration of 500 mL Ensure Plus®) only traces of bile salts have been reported (60 μM) [13]. Similar bile salt concentration (51 μM) was measured in the fed state stomach of healthy subjects after a standard lunch (13.5 g protein, 18 g corn oil, and 53 g carbohydrate in 300 mL water) [25].

1.2.3. Proteins, lipids and carbohydrates in gastric contents in the fed state

The protein, lipid and carbohydrate content in the fed state stomach is dependent on the type of meal consumed before the administration of the drug; therefore, their concentration is highly variable and cannot be expressed solely by the results of a single study. Indicatively, the concentrations of proteins and carbohydrates after administration of 500 mL Ensure Plus® to healthy subjects were found to be 23.3 mg/mL and 152.1 mg/mL at 30 min respectively, decreasing to 11.2 mg/mL and 49.1 mg/mL at 210 min after the liquid meal's administration [13].

1.2.4. pH of gastric contents in the fed state

The pH affects dissolution and absorption of both actively and passively absorbed drugs. The non-ionised fraction of the drug is more efficiently absorbed during passive absorption, while the affinity of the drug carrier for the ionised or non-ionised fraction defines the rate of active absorption [26, 27]. The pH of the stomach in the fed state is significantly higher than in fasted state (pH ≈ 1.7) [13, 28] with a wide range of values between 3 and 7 [1]. The pH increases up to approximately a value of 6.5 after a meal and decreases exponentially reaching a value of 2-2.7, similar to the pH value measured in the fasted state after 3-4 h. In case of patients suffering from hypochlorhydria/achlorhydria due to pathological conditions (i.e. AIDS; Acquired Immune Deficiency Syndrome) or administered medication (H2 receptor

antagonists or Proton-Pump Inhibitors), initial fasted pH values are elevated compared to the values mentioned above, reducing the dissolution rate of basic drugs [29, 30]. Thirty minutes after administration of 500 mL of a nutrient drink (Ensure Plus®), Kalantzi et al. reported a pH value of 6.4 in the gastric aspirates of twenty human subjects (Figure 1.1) and a decrease in the gastric pH to a value close to the fasted state three and a half hours after the liquid meal's administration [13]. The gradual decrease in gastric pH values is attributed to the induction of secretion of gastric acid after the administration of a meal and to the meal's buffering properties [22]. Another *in vivo* study [21] showed that after administration of a liquid standard meal containing 65% fat, 29.5% carbohydrate and 5% protein, the pH reaches a maximum of 4. A study by Yamaguchi et al. [31], which monitored the gastric pH of human subjects using a Bravo® pH monitoring system, with the aid of a capsule placed on the gastric wall, confirmed also the immediate burst and gradual decrease to the fasted state level pH, with it returning to its initial value 2 h after the administration of a meal. The subjects of the above study were monitored for 48 h and did not follow any restriction in their dietary routine. The absolute values of these two studies cannot be compared though, as the subjects of the latter did not follow a specific diet. The time needed for the gastric pH levels to return to the initial values and the pH "peak" value of the fed state are dependent upon the type of standard meal administered during each *in vivo* study, the age of the subject and the experimental protocol followed [32]. For instance, the pH decreases to the fasted state value faster after the administration of a liquid meal than after a solid meal. Gastric pH mostly affects the dissolution of drugs with a pKa value close to the physiological pH values, as when ionised behave as week electrolytes with their solubility being increased in comparison with their un-ionised form [27]. Therefore, changes in gastric pH mostly affect weak acids and weak bases with the increased values in the fed state enhancing the dissolution of acids and reducing the dissolution of bases. Gastric pH can also affect drug release. Coatings with pH-dependent disintegration properties, such as enteric coatings which dissolve rapidly in pH values of 4.5–8 [21] may experience different disintegration profiles due to elevated fed pH value.

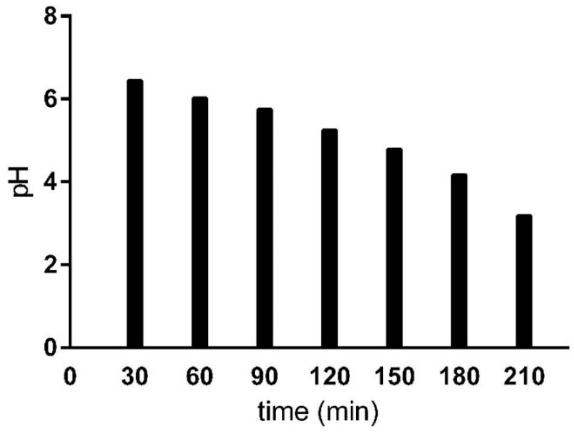

Figure 1.1. Mean pH values from aspirates of patients after administration of 500 mL Ensure Plus® containing 10 mg/mL PEG 4000. Data extracted from [13].

1.2.5. Osmolality of gastric contents in the fed state

Osmolality can affect drug's dissolution rate by inducing changes in the swelling behaviour of the formulation. Osmolality is linked with water penetration in the formulation; when the difference in osmotic pressure between the inner and outer (GI environment) part of the formulation decreases, water penetration decreases as well, affecting negatively drug release [33]. The gastric fluids in the fed state are slightly hyperosmotic 30 min after administration of Ensure Plus® (559 mOsm kg^{-1}), and their osmolality is decreased to 217 mOsm kg^{-1} 3.5 h after the administration [13], revealing that the osmolality of the gastric contents returns to the fasted state value during this time period (191 ± 36 mOsm kg^{-1} based on measurements of 24 healthy subjects) [12].

1.2.6. Surface tension of gastric contents in the fed state

The surface tension of the fed gastric fluids is lower than the one of an aqueous solution due to the presence of surface tension lowering compounds, such as bile salts entering the stomach through duodenal reflux and acting as surfactants, pepsin and food components [34]. Pepsin is an enzyme produced in the mucosal lining of the stomach and acts as a digestive protein in the gastric environment. Since the lowest surface tension values acquired after the addition of biorelevant concentrations (0.003–0.195 mg/mL) of the enzyme (fasted state) in an acidic solution (pH 1.6, HCl solution with 2 g/L NaCl) were 57 mN/m, it can be assumed that other surfactants are present as well in both fasted and fed state gastric fluids, as their surface tension value was significantly lower with a value of about 30–31 mN/m in the fed state and 33–43 mN/m in the fasted state [4, 35, 36].

1.2.7. Buffer capacity of gastric contents in the fed state

The buffer capacity of the medium can have a great effect on drug's dissolution in combination with its pH, as change in pH can affect the ionisation percentage and consequently the solubility of ionisable drugs and excipients. In the fasted state, bicarbonate is the buffer mainly present in the stomach [37]. In the fed state, the buffer capacity is dependent mainly on the meal contents than on the stomach's mucosa, making this chemical property highly meal-dependent [37]. Buffer capacity at gastric fed state conditions after administration of Ensure Plus® is around 14–28 mmol/L·DpH based on *in vivo* measurements [13]. Table 1.1 summarises the physicochemical properties of the contents of the gastric fed state environment, as measured *in vivo* in human subjects.

Table 1.1. Physicochemical properties of the contents of the fed state stomach based on *in vivo* measurements.

	Value	Technique employed	Meal	Sample	Reference
pH	6.23–6.84 (during meals and 1-2 hours after meal administration)	Bravo wireless pH monitoring capsule	No restriction in meal composition	11 volunteers (8 healthy)	[31]
	median 6.4–2.7 (from 5 min to 3h 30min)	Aspiration through nasogastric tube	500 mL Ensure Plus®	20 healthy volunteers	[13]
	median 5.0 (peak 6.7)	Heidelberg capsule	Standard meal 1000 kcal	34 healthy volunteers	[28]
Osmolality	559 mOsm kg^{-1} - 217 mOsm kg^{-1}, (from 30 to 210 min)	Aspiration through nasogastric tube /freezing point depression	500 mL Ensure Plus®	20 healthy volunteers	[13]
Buffer capacity	14-28 mmol L^{-1} DpH (from 30 to 210 min)	Aspiration through nasogastric tube /titration with HCl	500 mL Ensure Plus®	20 healthy volunteers	[13]
Surface tension	30-31 mN m^{-1}	Aspiration through nasogastric tube /titration with HCl	500 mL Ensure Plus®	20 healthy volunteers	[13]

1.3. Drug properties that relate to potential food effect

Food effects can be induced via the direct interaction of drugs, due to their unique physicochemical properties, with food components [38]. Such interactions include formation of insoluble complexes (i.e. tetracyclines and calcium ions) [39], binding to proteins (i.e. phenytoin) [40], or interaction/exchange of drugs with anionic or cationic sites of dietary fibres (i.e. metformin) [41]. In this section the drug properties that can affect drug dissolution and absorption leading to a potential food effect are described.

1.3.1. Ionisation (pKa)

The pKa determines the percentage of a drug's charged/uncharged form under certain pH conditions and affects the solubility of drugs at differing media pH. Solubility and dissolution of weekly acidic drugs are low at the pH of the fasted stomach as they are mostly in their unionised form. At fed state conditions, where the pH is higher, their gastric solubility and dissolution increase with a subsequent effect on their pharmacokinetics [6]. The uncharged state of the drug has a positive effect on membrane permeability, as the fraction of the unionised form of a drug is proportional to its lipophilicity [42]. As far as weekly basic drugs are concerned, their gastric solubility and dissolution are lower due to the higher pH of the stomach in the fed state in comparison with the fasted state. For compounds that are non-ionisable in the gastric environment a gastric fed state dissolution test is essential [6], as the type of meal consumed affects the surface tension of the gastric contents and thus, the active surface area that is available for drug solubility and dissolution [43].

1.3.2. Lipophilicity (log P, log D)

Partition coefficient, log P, is indicative of the lipophilicity of a compound and determines the partition of a compound in a system of n-octanol/water. For an ionisable molecule the apparent partition coefficient (log D) is the value which expresses the partition in the aqueous and organic phase in a more accurate way as it takes into consideration its ionised/unionised percentage and therefore log D values vary according to the pH of the environment. Log P values are related to drug's affinity for biological membranes and target sites affecting its biological activity [42]. Ideally, the drug should have such a hydrophilic-lipophilic balance so that it can be dissolved in the biological fluids, where the site of absorption is, and also be able to permeate the membranes of the site of action. Drug's lipophilicity is believed to have an important role in its dissolution in the gastric fed state, as solubility and

dissolution of lipophilic drugs in the fed stomach are performed through their partitioning in the lipid fraction of the meal during its breaking into particles throughout digestion before reaching the small intestine [6].

1.3.3. Solubility

Solubility is a key drug property for its potential oral absorption. Due to the prolonged residence of the drug in the gastric environment during the fed state, the solubility in the gastric contents will affect drug's dissolution and subsequent absorption. The wetting and solubilisation of drugs co-administered with food can be increased by the digestion products of lipolysis products in the gastrointestinal tract (Figure 1.2) [22]. The gastric compartment is the part of the gastrointestinal tract where the dietary lipids are emulsified at first place. Facilitated by gastric lipase, gastric agitation and emptying, protein and polysaccharide contents along with lypolytic triglyceride products stabilize the interface between lipid and aqueous phase [18]. Therefore, the presence of gastric lipase in the fed stomach is important when biorelevance needs to be achieved for *in vitro* assays. Aqueous media usually give an underestimation of the drugs' solubility in the gastric environment. An underestimation of solubility was also observed for undigested milk, suggesting that apart from having the same pH, buffer capacity, protein content and osmolality, the presence of enzymes should be considered for a good prediction of drug's solubility in the gastric environment [44]. If not adequately soluble in the gastrointestinal fluids, drugs orally administered can have a solubility limited dissolution and nonlinear dose responses due to inadequate drug in solution in the site of absorption [42].

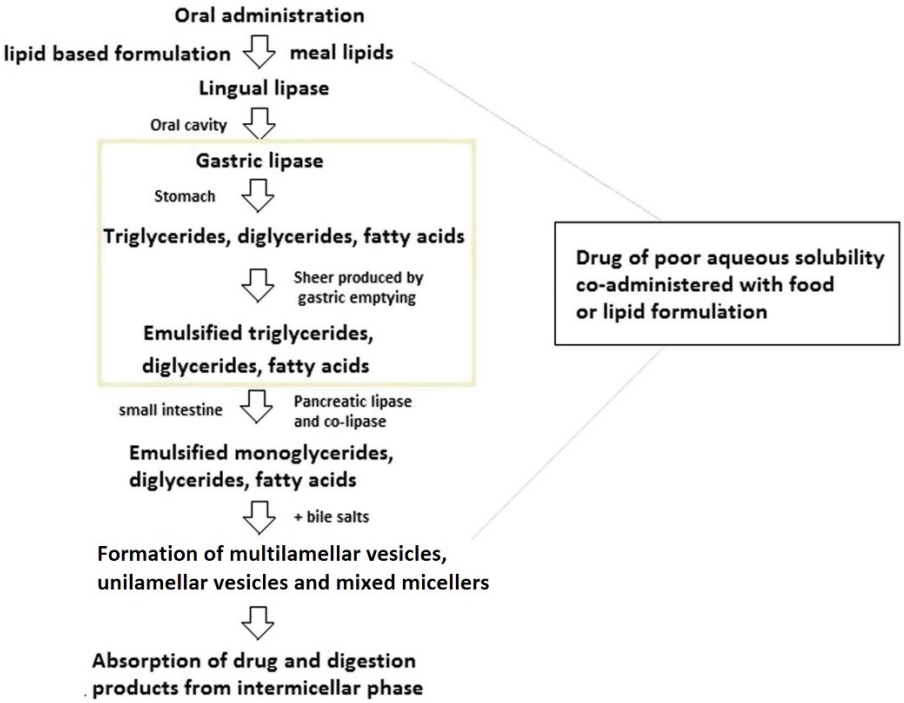

Figure 1.2. Schematic representation demonstrating the lipid digestion, formation of micelles and drug absorption in the small intestine after administration of a meal; processes taking place in the stomach in the highlighted rectangle. Modified from [22].

1.3.4. Biopharmaceutics Classification System (BCS) and food effect

Amidon and co-workers [45] defined drug aqueous solubility and permeability as determining parameters which control a drug's systemic *in vivo* absorption and introduced the Biopharmaceutics Classification System (BCS) which categorises the drugs in four classes according to their aqueous solubility and permeability:

BCS I compounds: high solubility-high permeability

BCS II compounds: low solubility-high permeability

BCS III compounds: high solubility-low permeability

BCS IV compounds: low solubility-low permeability

Fleischer et al. [46] proposed a food effect predictive model for the drugs' absorption according to their drugs' BCS class (delayed/no effect for BCS class I, increased with fat content for BCS class II, decreased for BCS class III, low and non-predictable for fed/fasted states for BCS class IV); this model is only a general guideline as many drugs do not follow this pattern. The reason is that except for BCS class I drugs, the drugs belonging to the other

BCS classes have a wide range of properties and consequently different rate limiting steps for drug absorption. For example, a low solubility compound with absorption just under 90% is classified as a class IV compound, but it is unlikely that its permeability would be the rate limiting step for its absorption [47]. Based on the BCS classification, Wu and Bennet [48], correlated the interactions of the different BCS class drugs with intestinal efflux and influx transporters. It was suggested that for BCS class II compounds, the relative magnitude of the inhibition between efflux and influx transporters with additional solubilisation in the intestinal environment and gastric emptying are the two parameters affecting the drugs' absorption, with the latter process being the determinant one. Inhibition of uptake transporters was suggested as a possible reason for negative food effect for most BCS class III compounds, while BCS class IV compounds combine all the above mechanisms for BCS classes II and III drugs (Table 1.2) making the prediction of food effect difficult [49].

Table 1.2. Biopharmaceutics classification system, predictability of food effect and transporter effect [46, 48].

BCS class	Solubility/ permeability	Food effect	Drug examples	Transporter effect on drug disposition	Transporter effect by high fat meal
I	+/+	no effect	Disopyramide Ketoprofen Verapamil	Minimal	No effect
II	-/+	+	Cyclosporine Danazol Dapsone	Efflux transporter effects predominate	Efflux transporters inhibition, intestinal drug solubilisation (drug passively absorbed) Inhibition of both absorptive and efflux transporters. Food effect according to relative inhibition (drugs actively absorbed)
III	+/-	-	Fesofenadine Nadolol Valsartan	Absorptive transporter effect predominate	Inhibition of absorptive transporters in the intestine
IV	-/-	+, -, no effect	Chlorothiazide Furosemide Neomycin	Possible substrates for both absorptive and efflux transporters	All effects mentioned above for classes II and III

1.4. Standard meals used in BA/BE studies

Homogenised standard meals have been used as an attempt to simulate gastric fed state conditions. In order to determine the effect of food on drug absorption, both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) recommend the use of a high-fat meal for the determination of drug's pharmacokinetic parameters in the fed state as the worst-case scenario [50]. Meals that are of a high caloric and fat content are recommended in BA/BE studies as these are more likely to affect gastric physiology and have a more pronounced effect on drugs [51]. As a high-fat and high-calorie meal FDA suggests a meal of 800–1000 caloric content with ~ 50% of the calories deriving from its fat content with 150, 250 and 500–600 kcal being obtained by protein, carbohydrate and fat, respectively [51]. An example of a typical high fat standard breakfast as proposed by the FDA used in a bioequivalence study for Cicloral® and Neoral® (100 mg cyclosporine A formulations) [52] is as follows: "2 eggs fried in butter, 2 strips of fat bacon, 120 g hash brown potatoes, 250 mL whole milk, and 1 croissant". For food effect studies, EMA suggests a similar standardised high fat meal (800–1000 kcal caloric content, 500–600 and 250 of which derive from fat and carbohydrates, respectively) and a moderate meal of ~ 400–500 kcal with ~ 150 kcal deriving from fat [50]. As far as the dosage strength to be tested in fed state studies is concerned, FDA recommends the testing of the highest dose to be marketed and lower doses if the testing of the former is not possible for safety reasons [51]. According to EMA, the highest and lowest doses in the drug therapeutic range have to be tested when the drug follows nonlinear pharmacokinetics [50].

Apart from the meals described previously, other types of meals can also be used in the investigation of the effect of food in drug's pharmacokinetics in cases of a specific food effect mechanism. For example, a high-protein meal (80 g protein, 52 g carbohydrate, 9 g fat) was used in the investigation of the pharmacokinetics of gabapentin, an anticonvulsant, whose transport through the biological membranes is controlled by System-L, the l-amino acid transport system [53]. Klein et al. [43] characterised two standard meals (GSK high fat-(62%) standard meal and FDA intermediate fat (37%) standard meal), constituted by homogenised eggs, bacon, butter, milk and other ingredients indicative of a median diet (Table 1.3). The purpose of the study was the comparison of their physicochemical properties with those of the meals, such as milk and nutrient drinks currently being used for the simulation of fed-state conditions in dissolution studies.

Table 1.3. Examples of meals used for the determination of drug-food interactions *in vivo*.

Meal	Composition	Fat content	Protein content	Carbohydrate content	Reference
GSK high-fat standard meal	2 slices of toasted white bread with butter, 2 eggs fried in butter, 2 slices of bacon, 2 oz of hash browned (fried shredded) potatoes, 8 oz of whole milk	67 g (603 kcal, 62% of total calories)	33 g (132 kcal, 14% of total calories)	58 g (232 kcal, 24% of total calories)	[43]
FDA high-fat standard meal	2 eggs fried in butter, 2 strips of bacon, 2 slices of toast with butter, 4 oz of hash brown potatoes, 8 oz of whole milk (800–1000 kcal)	500–600 kcal	150 kcal	250 kcal	[51]
FDA intermediate-fat standard meal	1 English muffin with butter, 1 fried egg, 1 slice of cheese, 1 slice Canadian bacon, 1 serving of hash browned (fried shredded potatoes), 6 oz of orange juice, 8 oz of whole milk	27 g, (240 kcal, 37% of total calories)	29 g (116 kcal, 18% of total calories)	73 g (292 kcal, 45% of total calories)	[43]
Light standardised breakfast	Not specified (+100 mL of black coffee)	26 g	28 g	51 g	[121]
Standardised high-carbohydrate meal	Not specified (1000 kcal)	Not specified	Not specified	600 kcal	[122]
Low-fat meal	1 slice of white spread bread with jelly, 6 oz of orange juice, 8 oz of skim milk (250 kcal)	1 g	12 g	51 g	[83]

Long chain triglyceride meal	Fat: 31% w/w long chain triglycerides Proteins: whey, casein and soy hydrolysates Carbohydrates: monosaccharides, oligosaccharides, polysaccharides	36% of total calories	16% of total calories	48% of total calories	[123]
Medium chain triglyceride meal	Fat: 80% w/w medium chain triglycerides (C_6-C_{12}) Proteins: whey, casein and soy hydrolysates Carbohydrates: monosaccharides, oligosaccharides, polysaccharides	36% of total calories	16% of total calories	48% of total calories	[123]
High-protein meal	2% low fat milk, Carnation Instant Breakfast® and Pro Pac® Plus (protein supplement)	9 g	80 g	52 g	[53]
High-protein meal	Not specified (439.5 kcal)	17.1 g	30.5 g	43.5 g	[124]
Low-protein meal	Not specified (417.8 kcal)	14.9 g	10.5 g	64.3 g	[124]

1.5. *In vitro* simulation of gastric conditions in the fed state (biorelevant dissolution media)

Even though actual homogenised meals are able to simulate gastric state conditions the best, problems in the analysis of the drugs led to the development of alternative approaches [37, 54]. A range of dissolution media have been developed in order to simulate the *in vivo* conditions of the fed state stomach (Table 1.4). These media were developed with the aim of having the same physicochemical properties with the standard meal recommended by FDA for BA/BE studies [51].

Table 1.4. Physicochemical properties of gastric fed state biorelevant media used *in vitro*.

Medium	Mass (g)	Volume (mL)	Density (g/mL)	pH	Buffer capacity (mEq pH^{-1} L^{-1})	Osmolality (mOsmol kg^{-1})	Surface tension (mN m^{-1})	Viscosity (mPas)	Reference
Standard breakfast (62% fat*)	516± 6[a]	474±7.7[a]	1.09± 0.03[a]	6.51± 0.01[a], 6.61± 0.03[b]	29.3± 0.9[a], 30.1± 1.8[b]	771± 10	52± 1[a], 44± 1[b]		[43]
Standard breakfast (37% fat*)	540± 5.5[a]	513± 7.3[a]	1.05± 0.03[a]	5.28± 0.03[a], 5.12± 0.04[b]	49.6± 1.7[a], 47.2± 1.5[b]	713± 10	49± 1[a], 45± 1[b]		[43]
Milk (48.1% fat*)			1.03± 0.005[a]	6.72± 0.02[a], 6.63± 0.01[b]	14.4± 0.2[a], 13.9± 0.2[b]	285± 2.7	54.2±0.4[a], 49.8± 0.6[b]	1.9± 0.04[a], 1.5± 0.04[b]	[43]
Partially digested milk (i. 3.5% fat milk, ii. milk + HCl + pepsin, iii. Milk + HCl				i. 6.5, ii. 4.7-2.6 (in 6 h), iii. 5.1-4 (in 6h)	i. 13-19 ii. 19-38 iii. 47-69	i. 260, ii. 338 462, iii. 475-540			[44]

+ pepsin + lipase)									
Ensure® (30.1% fat)			1.04± 0.016[a]	6.68± 0.01[a], 6.58± 0.1[b]	15.4± 0.1[a], 16.4[b]	375± 3.5	50.5±0.2[a], 47.8± 0.1[b]	6.3± 0.09[a], 4.4± 0.07[b]	[43]
Ensure Plus® (29.1% fat)			1.08± 0.003[a]	6.62± 0.03[a], 6.45± 0.02[b]	20± 0.7[a], 21± 0.3[b]	730± 10	53.2± 0.2[a], 48.4± 0.1[b]	19.1± 0.1[a], 12.3± 0.1[b]	[43]
Early FeSSGF (milk-based)				6.4	21.33	559			[37]
Middle FeSSGF (milk-based)				5	25	400			[37]
Late FeSSGF (milk-based)				3	25	300			[37]
Early FeSSGF (Lipofundin® -based)				6.4	21	559			[66]
Middle FeSSGF (Lipofundin® -based)				5	25	400			[66]
Late FeSSGF (Lipofundin® -based)				3	25	300			[66]

Nutrison®				5.4		420			[5]
FSGES				5					[125]
Intralipid® 30%						320		2.7± 0.06	[126]

[a] 25 °C.

[b] 37 °C.

[c] Calories derived from fat.

1.5.1. Milk-based media

1.5.1.1. Milk

Milk started being used as a dissolution medium for gastric fed state simulation about twenty years ago. Macheras et al. successfully used low fat milk (0.75% fat) with a flow injection serial dynamic dialysis technique (FISDD) as a food simulation medium for drug dissolution, for four drugs of different physicochemical properties: salicylamide, acetaminophen, propantheline and nitrofurantoin. Milk was selected as a dissolution medium in this study due to its potential as substrate of the gastric fed conditions and also due to its use as a vehicle in drug delivery systems [55]. Furthermore, its energy content is similar to that of a standard meal administered to the subjects participating in bioavailability/bioequivalence studies [56]. Despite its similarities with the gastric environment in the fed state, the use of milk does not always simulate the gastric fed state ideally. The issues of the use of milk as a dissolution medium relate to its lower values in osmolality (285 ± 2.7 mOsm kg^{-1}) and buffer capacity (13.9 ± 0.2 mEq pH^{-1} L^{-1}) at 37 °C compared to the standard high-fat breakfast proposed by the FDA (771 ± 10 mOsm kg^{-1} and 30.1 ± 1.8 pH^{-1} L^{-1}, respectively) [43]. Other issues relate to its higher pH value (pH ≈ 6.5) than the equivalent pH of gastric media after a meal (5.8 ± 0.2 after 50% of gastric emptying after liquid meal administration) [57], and the possible need of supplementary enzyme addition due to the digestion of milk's lipids and proteins taking place *in vivo* [6].

In 1998, Galia et al. [58], assessed the suitability of full fat milk as a biorelevant gastric fed state medium for the evaluation of the dissolution behaviour of one BCS class I drug (acetaminophen) and two BCS class II drugs (danazol and mefenamic acid). The results of this study demonstrated that for BCS class I drugs there is a strong dependence between the absorption and the type of formulation, with the interaction between the fed matrix and excipients controlling the absorption rate. Milk's high content in lipids enhances the solubility and dissolution of lipophilic drugs; for instance, release of danazol, a BCS class II drug, in milk, was substantially higher than in water. Furthermore, the pH of milk (pH ≈ 6.5) favours the dissolution of weak acids, such as mefenamic acid [58]. Diakidou and co-workers showed that despite milk's similarity in pH and protein contents with human aspirates after administration of a liquid meal, the solubility values of two BCS class II weak bases [dipyridamole (pKa 5.7–6.4, log P 2.7) and ketoconazole (pKas 2.9, 6.5; log P 4.4)], were 4.7

and 3.6 times lower in milk (after the addition of pepsin and lipase from Rhizopus niveus) than in the gastric fed-state aspirates, respectively [44].

1.5.1.2. Digested milk

After administration of a meal *in vivo*, digestion takes place. *In vitro* digestion milk models have been used for simulation of the *in vivo* digestion of gastric contents. These *in vitro* milk based models, take into consideration the role, amount and activity of the physiologically existing gastric enzymes in the fed state.

In a milk based medium, HCl, lipase and pepsin have been added [44, 59, 60]. Two models using bovine milk were considered for the simulation of gastric environment. In the first model the dissolution of l-sulpiride, a hydrophilic weak base was studied in milk digested with pepsin and HCl [60], with 4.4 mg of pepsin from hog stomach dissolved in HCl being added every 15 min for a 90-min time period. The dissolution assay was performed in USP Apparatus 2 (100 rpm, 500 mL volume). The second model used milk digested with pepsin/HCl/lipase from Rhizopus niveus aiming to simulate the fed gastric environment after food intake [44, 59]. In solubility studies of two lipophilic bases, dipyridamole and ketoconazole, it was shown that milk digested with the HCl solution of pepsin gave a good prediction of the ketoconazole's solubility in human gastric aspirates after administration of 500 mL of Ensure Plus® while a solubility overestimation was observed for dipyridamole. When milk digested with pepsin/HCl/lipase was used the prediction of the drug's solubility in the gastric aspirates was dependent on the time that the *in vivo* sample was collected [44]. The biorelevance of the addition of lipase in the milk in terms of pH and protein content was shown in a release study of felodipine from an extended release matrix. Gastric pH decreased slower and protein content faster than an identical medium in the absence of lipase, giving pH and protein content values closer to the ones observed *in vivo* [13].

1.5.1.3. Fed State Simulated Gastric Fluid (FeSSGF)

As an effort to improve the biorelevance of milk as dissolution medium and simulate the postprandial conditions of the gastric tube, a medium called Fed State Simulated Gastric Fluid (FeSSGF) was developed. This medium was developed by Jantratid and his co-workers [37] and is comprised of 3.5% fat milk diluted with acetate buffer. In order to mimic the three phases of gastric digestion with the pH value being 6.4, 5.0 and 3.0 for the early, middle and late phases, respectively, a FeSSGF for each phase was prepared (Table 1.4) [37]. The pH was adjusted with the use of 0.1 N HCl and NaOH solutions and it was suggested that the "middle"

medium reflects in a satisfactory manner the sum of the physiological gastric conditions during meal ingestion. Accepting this compromise, FeSSGF could potentially be used as a universal medium potential for fed-state gastric dissolution [37]. It should be noted though that this medium does not contain any enzymes, so the presence of the gastric pepsin and lipase is not taken into account. As milk in the absence of enzymes can only simulate the gastric fed state condition in its early phase [8], the use of the three "snapshot" media can simulate the intraluminal changes in pH, osmolality and protein contents accurately.

1.5.2. Nutrient drinks/emulsions

The use of various nutrient drinks and emulsions for parenteral administration has been employed to studies as an attempt to mimic the gastric stomach conditions in the fed-state, as they have similar composition (Table 1.5) to the standardised meals used in drug food effect studies [6].

Table 1.5. Composition of nutrient drinks/parenteral emulsions used as fed state gastric media in *vitro*.

Nutrient drink	**Composition (per Litre)**[a]
Scandishake® Mix	598 kcal, 30.4 g fat, 11.7 g protein, 69.5 g carbohydrate[b]
Ensure®	930 kcal, 25 g fat, 38 g protein, 135 g carbohydrate
Ensure Plus®	1500 kcal, 46 g fat, 55 g protein, 210 g carbohydrate
Nutrison®	1000 kcal, 39 g fat, 40 g protein, 123 g carbohydrate
Intralipid® 30	3000 kcal, 300 g fat

[a] According to the manufacturer [63] (Ensure® and Ensure Plus® vanilla flavour).

[b] 85 g powder in 240 mL whole milk.

Scandishake® Mix is a nutrient drink used for the simulation of gastric environment in the fed state. It was used in the form of powder mixed with whole milk with simulated gastric secretions containing lipase and pepsin in a dynamic *in vitro* system (TNO TIM-1), simulating the stomach and small and large intestines' environment. Scandishake® Mix was used for the simulation of a high-fat meal in the development of a dissolution model for fosamprenavir (prodrug of the antiretroviral amprenavir) [61]. Food-induced disintegration of fosamprenavir's tablets was assessed using Magnetic Resonance Imaging (MRI). The stomach compartment in the dynamic system was comprised of two units separated by a silicon wall with the surface between the outer and inner tubes being thermostated (37 °C). The simulated gastric contents were mixed by application of water pressure to the walls, causing three

"contractions" and "relaxations" per minute. Simulated gastric lipase and pepsin were pumped to the compartment at a flow of 0.5 mL/min. The nutrient drink, compared with simulated gastric fluid (SGF) which was used for the gastric fasted state simulation, predicted the formulations' postprandial delay in disintegration observed *in vivo*. This effect on the tablet's disintegration can be attributed to the competition of the nutrient drink with the water molecules for the interaction with the matrix and the formation of a layer of increased viscosity around the tablet [61].

The nutrient drinks Ensure® [62] and Ensure Plus® [13] have been used in several studies as biorelevant fed–state gastric media. According to the manufacturer [63], both emulsions contain water, corn maltodextrin, sugar, milk protein concentrate, canola oil and corn oil (Ensure Plus®) or soy oil and sucromalt (Ensure®). Intralipid®, an emulsion of similar fat content with Ensure® and Ensure Plus®, has also been used for the simulation of gastric fed state conditions after the administration of a high-fat breakfast [10]. Ensure® contains fat (3.7%), protein (3.7%) and carbohydrate (14.5%), while Intralipid® is available in 10, 20 and 30% fat concentrations (soya oil), with the emulsion also containing egg lecithin protein (12 g/1000 mL) and glycerol (22, 22 and 16.7 g/1000 mL for 10, 20 and 30% fat, respectively). Ensure® and Intralipid® 10% were used at a 1:6 dilution with universal buffer (pH 2.5 and 5.6) in dissolution (USP Apparatus 2, 100 rpm) and microcalorimetry studies of aminophylline controlled release tablets comprised of an aliphatic alcohol and hydroxyalkylcellulose [64]. Each dissolution profile was the expression of two distinct processes: dissolution of the alcohol and diffusion of the drug through the cellulose derivative. The profile was comprised of two first order rates separated by a mixed rate. The composition of the biorelevant medium mainly affected the rate of the initial first-order process and also the onset time of the second apparent first order, indicating that differences in the medium can affect the drug release mechanism.

The similarities of the physicochemical properties (pH, osmolality, buffer, capacity, viscosity and surface tension) between standard meals used in *in vivo* studies and gastric fed state media used *in vitro* have been assessed [43]. Ensure Plus® demonstrated better resemblance to the gastric conditions after the administration of a homogenised standard breakfast than milk and Ensure® but had significantly different viscosity, an issue resolved after addition of 0.45% w/v pectin, a water soluble polysaccharide. *In vitro* dissolution studies performed with Ensure® (USP Apparatus 2) [62] and Ensure Plus® [64] (USP Apparatus 3) demonstrated that dissolution behaviour is greatly affected by interaction between medium components and formulation. In the above studies, food effect observed *in vitro* was attributed

to a formation of a hydrophobic layer around the formulation (HPMC matrix) decreasing water ingress in the tablet [64], or by granting a more effective hydration of a matrix component [62].

Another nutrient drink, Nutrison®, was used for the simulation of gastric fed state conditions in dissolution studies (USP Apparatus 2, 100 rpm) for the study of possible food-drug interactions between food components and metoprolol tartrate IR tablets [5]. The nutrient drink contains 6 g/100 mL protein, 6 g/100 mL vegetable oil fat, 18 g/100 mL carbohydrate, vitamins and minerals. For its use as a biorelevant medium it was diluted to 60% with a solution containing HCl, NaCl, KCl and sucrose, giving values of pH (pH = 5.4) and osmolality (420 mOsm kg^{-1}) similar to the *in vivo* fed state conditions. A potential excipient dependent mechanism of delay in tablet disintegration was indicated through the formation of a protein film from the medium's components around the tablets (visual observation) attributed to electrostatic or hydrophobic interactions between the proteins and the excipient confirmed by scanning electron microscopy (SEM). The dependence of the formation of this layer on certain tablet excipients was confirmed by preparation of single excipient-medium mixtures and observation of a precipitation layer in the vessel for each mixture. The effect of meal type was further evaluated by comparing tablet disintegration times in media containing a single (protein, fat or carbohydrate) or mixtures of the mentioned components present in the fed state medium. The presence of proteins increased the tablet disintegration time the most, with a more profound effect when proteins were combined with fat, carbohydrates or both.

Even though the composition of nutrient drinks such as Ensure Plus® is more similar to the high-fat meals administered in BA/BE studies, milk based media can simulate the fed gastric content taking the presence of secretions into consideration [65]. Moreover, with the two approaches mentioned before (gradual digestion and "snapshot" media), the changes in the fed gastric environment during time can be more closely simulated.

Another version of the FeSSGF "snapshot" media has been recently proposed replacing milk with Lipofundin® MCT 20 [66], an emulsion containing medium chain triglycerides, which administered parenterally to patients, provides essential fatty acids. The emulsion:buffer ratios were different to those of the milk based FeSSGF "snapshot" media (17.5, 8.75 and 4.375% v/v content for early, middle and late media, respectively compared to 100, 50 and 25% for the milk-based media). Lipofundin® does not contain any proteins, which facilitates the drug's analysis in the medium and has similar fat content to the fed stomach aspirates [67].

1.6. Drug and formulation-related food effect

Under fed state conditions, tablet disintegration is generally delayed. For example, as mentioned previously, a nutritional drink used both in *in vivo* (canine) and in *in vitro* studies delayed disintegration and dissolution of metoprolol tartrate tablets by creating the formation of a food-induced thin layer around the tablet which did prevent not only the water penetration in the tablet but also the drug particles from leaving the matrix [5].

Food can have a significant effect on the absorption of drugs. This can be affected by differences in the interaction of the active pharmaceutical ingredient and/or the formulation with the fasted and fed state environments. Examples of two drugs (itraconazole and nifedipine) demonstrating drug-related and formulation-related gastric food effect, respectively, are discussed below.

Itraconazole, an antifungal agent, is a well-studied drug in terms of its food effect. The positive effect of food on itraconazole's absorption has been verified by both *in vivo* and *in vitro* studies. An *in vivo* study on itraconazole capsules (2 × 100 mg capsules administered) containing sugar coated pellets in healthy subjects was performed with the use of the FDA standard breakfast for the determination of the drug's food effect [68]. The study showed a significant increase for both C_{max} and $AUC_{0\rightarrow\infty}$ values [(C_{max}(fasted) = 0.59 C_{max}(fed), $AUC_{0\rightarrow\infty}$ (fasted) = 0.61 $AUC_{0\rightarrow\infty}$ (fed)] (Figure 1.3) with the increase in the drug's absorption being attributed to the drug's increased solubility in the food components. A similar study by Zimmermann et al. [69] (one 100 mg capsule administered with a standard breakfast) demonstrated similar results, with the relative bioavailability in the fasted state being 0.54 times the one observed after meal administration. The differences were attributed to the high fat content of the meal and also to the longer gastric retention time in the fed state.

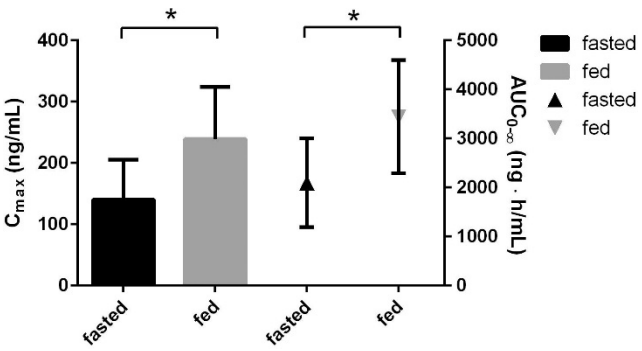

Figure 1.3. Pharmacokinetic parameters after administration of two 100 mg itraconazole capsules under fasted and fed (standard breakfast) state conditions. ∗ denotes a statistically significant difference ($p < 0.001$, multivariate ANOVA). Data extracted from [68].

In vitro studies were in agreement with the fact that the drug's bioavailability could be significantly affected by food [70]. An *in vitro* study used milk of different fat concentrations mixed with simulated gastric fluid (SGF) (pH adjusted to 3) as fed state biorelevant dissolution media and demonstrated that except for fat, other food components may be responsible for the food effect observed with itraconazole *in vitro*. Despite a pronounced difference between dissolution rates in the fasted (SGF pH 3) and the fed state simulated media, the fat content did not lead to statistically significant dissolution rates among the milk-based media. The presence of different carbohydrates (1% w/v glucose, lactose and starch in SGF) increased the dissolution rate at a small extend, possibly by formation of hydrogen bonds between the drug and the carbohydrates' hydroxyl functional groups. On the contrary, increased protein content, appeared to have a positive effect on drug dissolution. Drug dissolution rates in media containing albumin (0.5–4% w/v) concentration in SGF, increased by increasing the protein content. One possible mechanism could be through protein-drug binding possibly by the development of electrostatic and lipophilic interactions between the drug and albumin.

Nifedipine is a calcium channel blocker with a complicated formulation-related food effect [5]. Immediate release tablets, prolonged (modified) release tablets, capsules and soft capsules of nifedipine (5–60 mg) are commercially available [71]. Significant differences in the bioavailability of nifedipine modified – release formulations after oral administration have been observed *in vivo* between fasted and fed state conditions [72, 73]. The bioavailability of the brand formulation, Adalat® OROS (Osmotic-Release Oral System), has not been significantly affected by the presence of food [72, 74, 75]. Dose dumping and unusually long lag times, possibly due to lack of robustness of the mechanism of drug release in the fed state or due to prolonged stay of the formulation in the stomach, have been observed after administration of generic nifedipine formulations. During the gastric residence of nifedipine, the rate of absorption is limited, which led to rapid and high absorption when it reached the small intestine, altering the formulation's controlled release mechanism characteristics of some generic formulations [76, 77]. Nifedipine Sandoz® retard 30 mg (eroding matrix system) [74], Nifedicron® 60 mg, (capsule with mini-tablets, Pharmatec International, Milano, Italy) [75], Slofedipine® XL 60 mg (eroding matrix system) [72] and Nifedipine ER 90 mg test tablet (hydrophilic matrix, pilot formulation, Astra AB, Sweden) [78] demonstrated a formulation induced food effect after co-administration with a high-fat standard breakfast. Nifedipine Sandoz® retard demonstrated significant differences in its pharmacokinetic behaviour between fasted and fed states, possibly due to the inability of the matrix to release the drug in a

controlled way, with the exact mechanism not having been experimentally proven [76]. Dose dumping and a threefold increase of the C_{max} were observed for Nifedicron® under the fed state conditions compared to the fasted state [75]. Slofedipine® XL's profiles between the fasted and the fed state (high-fat breakfast) were also significantly different. Nevertheless, even though the geometric $AUC_{0\rightarrow 24}$ mean in the fed state was approximately half the one in the fasted state for Slofedipine® XL, the $AUC_{0\rightarrow tn}$ values were identical [72]. Similar behaviour was observed with Nifedipine ER tablets (90 mg), which demonstrated a higher absorption rate than Procardia® XL (90 mg) (osmotic pull-push system tablet), which was used as a reference product. The effect was attributed to the increased erosion rate as a result of the gastric motility and alterations in the gastric content after meal administration [78].

It is worth mentioning that in several of the above studies, differences in the fed state-pharmacokinetic behaviour between the brand and generic formulations have also been observed. In the case of nifedipine Sandoz® retard, great inter-patient variability often accompanied with fast, uncontrolled drug release was observed in plasma concentration vs time profiles, demonstrating failure of the controlled release behaviour of the formulation under fed conditions [74]. In case of Slofedipine® XL [72], the authors concluded that the differences between the test (Slofedipine® XL) and the reference (Adalat® OROS) formulations in the fed state could be attributed to the prolonged transit time of the former. Slofedipine® XL had a significant delay on the onset of its therapeutic action in 15 out of 24 patients of the study (15 h lag time), which resulted in 29% decreased $AUC_{0\rightarrow tn}$ compared to Adalat®. Slofedipine® XL's lag time was attributed to the fact that undissolved particles of the formulation of a diameter above 10 mm were not able to pass through the pylorus, until the onset of phase III of the migrating motor complex (MMC; the cylindrical series of gastric electrical activity, taking place between meals) [79]. In the same study, *in vitro* dissolution studies in acetate buffer pH 4.5 demonstrated that at pH values similar to the fed state, the generic formulation remained undissolved for 24 h, in contrast to Adalat® OROS which was almost 100% dissolved.

1.7. Meal-related food effect

Drug food effect relates to the nature of the meal. Meal characteristics such as fat content, viscosity, caloric content, size, and volume are parameters which can affect its absorption.

Increased fat content in the fed state relates to delayed drug absorption due to slower gastric emptying rate [23] and to decreased drug dissolution due to retarded wetting of the

formulation [80]. Increased viscosity of the gastric contents due to the administered meal delays the rate of gastric emptying [81]. Increased meal viscosity can decrease the diffusion coefficient of a compound according to Stokes-Einstein equation [82], which could in turn decrease drug absorption if it is only absorbed in a specific part of the gastrointestinal tract, as the drug goes past this site. The effect of meal viscosity is impaired in the small intestine due to secretions and digestion products. The most common effect of high meal viscosity is an increase in the T_{max} values of drugs. In an *in vivo* study using canine subjects, when a calorie free viscosity enhancer, HPMC (hydroxypropylmethylcellulose) was added, a delay in the T_{max} of the antiarrhythmic bidisomide was observed [83]. The meal's residence time depends also on its caloric content [26]. 2–4 kcal of caloric content per minute is transferred to the duodenum [17], meaning that meals with high caloric content will reside in the stomach for longer periods, affecting drug's transit time in the gastrointestinal tract. Nutrient drinks of 1 kcal/mL content are emptied at a rate of 2–2.5 mL/min, while nutrient drinks of 0.2 kcal/mL content have an emptying rate of 10 mL/min [17]. A calorie-dependent decrease in the gastric emptying rate was observed for the solid portion of the meal (45 ± 3.4% of the meal mass retained in the stomach for the 68 kcal solid meal and 65 ± 4% for the 633 kcal solid meal at 100 min), while its liquid portion is emptied to the duodenum at a rate independent of its energy content [11]. Meal size and volume relate to its gastric residence time [11]. Meals of fourfold mass and similar caloric content resulted in a 388% higher emptying rate, attributed to activation of stretch or volume receptors in stomach, increasing peristalsis. Using different volumes of isocaloric meals, a statistically significant increase in gastric emptying rate with the larger volume was noted for volumes of liquid meals between 200 mL and 800 mL [84].

1.8. Analytical techniques and challenges for sample treatment and drug quantification

Most of the gastric fed state media used until now are milk based or o/w emulsions; therefore, several sample preparation processes have been developed for the extraction of the drugs from these matrices. Milk is a complex biological fluid containing proteins, lipids, carbohydrates, divalent and trivalent cations which can be bound to the compounds of interest making drug analysis challenging. The analytical techniques, issues and challenges described in this section refer mainly to fed state gastric biorelevant media which are at least partly comprised of milk or contain other types of lipid and protein sources. The quantification of drugs in these media is usually performed using high performance liquid chromatography

(HPLC) and therefore, the analytical challenges and problems presented below refer mainly to this technique.

1.8.1. Filtration

The first challenge with drug analysis when biorelevant media are used is filtration itself. Filtration is an essential step in the analytical procedure, as biorelevant media contain a range of particles deriving from lipids, carbohydrates, fat and salts, which have to be removed before the sample's injection in the HPLC. Moreover, when fed biorelevant media such as milk or FeSSGF are used, the use of small pore size filters for the sample analysis during solubility or dissolution studies cannot be used due to clogging from the presence of large proteins [9]. Several types of filters such as 0.45 or 0.22 μm polytetrafluoroethylene (PTFE) [64] or regenerated cellulose (RC) [44] have been used for sample analysis prior to HPLC injection for drug quantification in both milk and lipid-based media but a sample clean-up step is required before for these type of media. Glass microfiber (GF) 2.7 μm filters have also been used in drug solubility and dissolution studies in milk based media [85], in order to remove undissolved drug or formulation particles prior to sample treatment and filtration through the filters of smaller pore size. Filters of bigger pore size, attached to the sampling cannulas of the USP Apparatus 1/2 (polyethylene sticks, 10 μm and nylon membrane filters, 5 μm) have also been used in dissolution studies with FeSSGF and milk for the same reason [56, 85].

Adsorption of the analyte on the filters should be studied in order to evaluate and choose the appropriate filters. Salicylic acid and sodium saccharine are example of drugs which demonstrate significant adsorption on nylon filters as shown in a study by Carlson and Thompson [86], where 85.8% and 60.4% of salicylic acid at 0.005 mg/mL and 0.05 mg/mL concentrations respectively were adsorbed on 25 nm nylon filters. Sodium saccharine demonstrated even higher adsorption with the entire drug (100%) being adsorbed on the same filters at a 0.1 mg/mL concentration [86, 87]. It should be noted that if the first few drops of the filtrate are not discarded, the percentage of adsorption can reach extremely high values.

1.8.2. Medium

The analysis of the drug content in milk based media can be challenging as the content of the medium itself is comprised of a lipid and an aqueous phase that requires separation of the phases before an HPLC analysis can be performed. Analysis could be affected by the differential distribution of the drug in the multiple phases of the milk based media, as it could distribute in the either aqueous or lipid phase or even bind to the proteins or fat contained in

the medium. Several drugs have shown binding in milk at amounts higher than 50%; diazepam, indomethacin, grizeofulvin and dicumarol demonstrated binding percentages from approximately 55–95% in low and full fat milk (37 °C) after equilibrium dialysis against a phosphate buffer solution (pH 6.5). For some drugs, their percentage bound was more significantly affected by milk's temperature (dicumarol, prednisolone) and fat content (e.g. binding of diazepam increased more than 13% in full fat milk in comparison with low fat milk at temperatures 15–37 °C) [88]. A factor affecting the analysis of milk is its variable fat, carbohydrate and lipid content. Its composition differs among different mammalian species, and is also affected by parameters such as their diet or the onset of their lactation period [89]. Therefore, milk of the same commercial brand and batch should be used when different drugs and dissolution conditions are compared, as changes in the medium composition may affect parameters such as recovery, precision, and analytical method compatibility.

1.8.3. Sample treatment and analysis

1.8.3.1. Protein precipitation (PP)

One simple method of sample clean-up prior to drug quantification in gastric milk-based fed state media is protein precipitation. Protein precipitation is the technique mainly used until now with the addition of a volume of an organic reagent to a volume of medium, followed by a centrifugation and a filtration step before its analysis in HPLC. The precipitation of the milk's proteins can be performed using an organic reagent followed by filtration and centrifugation steps [9].

A range of solvents such as acidified MeOH [90], acetone [91] and HCl [92] have been used as protein precipitation reagents for the extraction of drugs from milk-based media. Parameters such as their compatibility with the analytical technique chosen, their volatility in case organic phase evaporation is needed, their selectivity and their cost have to be considered during the selection of an appropriate protein precipitation solvent [88].

Fotaki et al. [60] suggested a precipitation and centrifugation method for the quantification of l-sulpiride, a BSC class III drug, in a milk based dissolution medium, proposing an assay involving centrifugation at 4000 rpm for 10 min (8 °C) for the separation of the aqueous phase from the lipid phase with acetonitrile being added in the aqueous filtrate (1:2) and a last centrifugation and filtration step (Titan® filters 0.45 μm) following. Sample treatment in nutrient drinks and emulsions requires a more time consuming clean-up procedure. Ensure Plus® requires phase separation via a centrifugation step (e.g. 11,500 rpm, 1.5 h) [64]

and possibly an extra filtration step with a larger pore size filter (5 μm) prior to filtering with a 0.45 μm filter and injecting the supernatant in the HPLC.

A study by Williams et al. [10] used 1:2 with ice-cold 12% (w/v) trichloroacetic acid for milk protein precipitation and then centrifugation at 10,000 rpm for 15 min extracting 0.2 mL of the supernatant for the HPLC analysis [10] for the quantification of caffeine in both milk-based (0.1%, 1.7%, 3.6% fat milk) and fat emulsion type [30% fat emulsion (Intralipid®)] media. Despite the fact that a protein precipitation step, followed by centrifugation and filtration, was adequate for both types of media, the recovery of the drug in the fat emulsion was significantly lower. A challenge associated with the selection of protein precipitation reagents is their compatibility with the HPLC method for the analysis of the selected drug. Peak fronting is a common issue when organic reagents of higher strength than the mobile phase are used: an issue that can usually be resolved with evaporation of the reagent under nitrogen and reconstitution in the mobile phase. Another disadvantage of this technique is its inability of complete removal of the lipid part of the medium [89], as for the selective removal of lipids, a supplementary step is required. This step may be a wash with hexane, given that the compound of interest is ionised or not extracted in hexane, so as not to be lost during the washing step [93].

1.8.3.2. Solid phase extraction (SPE)

Another method used for the extraction of the analyte of interest from milk is solid phase extraction (SPE). Solid phase extraction (SPE) is widely used for the extraction of drugs from biological fluids such as whole blood [94], plasma [95], urine [96] and milk [97], and is often preceded by a protein precipitation step. SPE cartridges are comprised of a polypropylene tube with their sorbent between two porous frits. A typical experimental protocol involves 4 main steps: (i) cartridge conditioning, (ii) sample loading, (iii) sample washing and (iv) sample elution [98].

Most cartridges are either comprised of bonded silica phases, similar to the material of the reversed phase HPLC columns but with bigger diameter particles (10–60 μm), or of polymeric resins (e.g. polystyrene-divinylbenzene) [99]. SPE C_{18} cartridges have been used for the quantification of several drugs, such as β-lactam antibiotics [100] or nonsteroidal anti-inflammatory drugs (NSAIDs) [101], in milk. In the above studies, a protein precipitation step similar to the one previously described [60] took place before the extraction, due to the milk's high content in proteins and lipids, which render the sample too "dirty" to be loaded straight

on the cartridge. An elution solvent of high water percentage is preferable for extraction from milk-based media, and has to be able to elute the drug and retain most of the lipids on the cartridge [10]. The extraction yield of lipid drugs using SPE may be low due to the drug's interaction with milk's fat globules. A way to disrupt this drug-fat globule interactions would be sonication and dilution of the medium before its loading on the cartridge [89]. A challenge for the analysis with SPE when it comes to biorelevant media, is the interference from the matrix in HPLC analysis. Therefore, clean-up with a solid phase extraction cartridge can help towards the development of more sensitive and robust methods in drug analysis in fed state biorelevant media. Disadvantages of SPE as a drug's extraction method from gastric fed state media include the quick drying of the cartridges and the difficulty to adjust the vacuum during the multiple steps without the presence of an automated manifold, affecting the reproducibility of the method [102, 103].

1.8.3.3. Liquid-liquid extraction (LLE)

Liquid-liquid extraction is based on the analyte partitioning between an aqueous phase and a water-immiscible solvent [99]. Several extraction protocols have been successfully applied for a wide range of drugs such as mycotoxins [104], mycrocyclic lactones [105], vitamins [106] and analgesics [107]. The main advantages of LLE is the short time required for method development and its low cost. A serious drawback is the fact that it is a time-consuming and labour intensive method. Moreover, the possible presence of the milk's lipid content in the extraction solvent after the LLE process, leads to phase separation of the sample and lipid partitioning in the stationary phase of the HPLC column during drug analysis. Therefore a washing step with hexane is usually required, so as to remove the lipids [89], making the whole procedure even more time consuming. The following protocol used in milk for the quantification of Ochratoxin A is a typical case of liquid-liquid extraction [104]; 0.2 mL saturated NaCl solution and 2.4 mL chloroform were added to 1 mL milk, mixed gently for 3 min, centrifuged (4500 rpm, 20 min) and after the removal of the chloroform layer, evaporation to dryness under nitrogen steam and reconstitution in acetonitrile followed. Lipid removal was performed by double extraction (2 × 0.4 mL petroleum ether for 1 min). After discarding the etheric layer, acetonitrile was blown to dryness, reconstituted in 1 mL of mobile phase, filtered and analysed in HPLC.

1.8.3.4. Ion selective Electrode (IsE) sensor

An online monitoring system, in an attempt to avoid the sample treatment traditionally required for the extraction of the analytes of interest from FeSSGF has been published [9]. An Ion Selective Electrode (ISE) sensor system was used, with two electrodes placed constantly in the dissolution vessel which were able to monitor the changes in drug concentration through the changes in potential. With this proposed methodology, diphenhydramine hydrochloride was successfully quantified in a dissolution study using the USP Apparatus 2 in several fasted [Fasted State Simulated Gastric Fluid (FaSSGF), Fasted State Simulated Intestinal Fluid (FaSSIF) and Fasted State Simulated Intestinal Fluid Version Two (FaSSIF-V2)] and fed (FeSSGF) state biorelevant media. Sample preparation steps needed with extraction techniques were avoided, and a continuous dissolution profile and a much faster and less laborious alternative were offered. Disadvantages of this method are as follows: (a) its limitation to the analysis of ionised drugs, (b) the complicated correction of the baseline needed for the heterogeneous biorelevant media and (c) its inability for the analysis of compounds of extremely low aqueous solubility [9].

1.8.3.5. Other techniques

Several other techniques have been used for drug quantification in milk and may have the potential to be used in fed state gastric media, some of which are briefly listed below:

Matrix solid-phase dispersion (MSPD)

The matrix solid-phase dispersion (MSPD) technique was firstly developed by Barker et al. [108]. MSPD involves the grinding of biological samples with sorbent particles producing a column material acting as a solid support from which the drugs in the matrix can be selectively extracted [109, 110]. MSPD has been successfully used in drug quantification in milk with sorbents, such as C_{18} [111], C_8, silica gel [112], mixed-mode/cationic-exchange (MCX), mixed-mode/anion-exchange (MAX) and weak anion–exchange (WAX) [113], and more recently molecularly imprinted polymers (MIPs) [110]. An advantage of this method is the combination of homogenisation, fractionation and purification in one single step and could be also used to milk-based gastric dissolution media [109]. It is also cheap and environmentally safe but relatively labour demanding [114].

Solid phase microextraction (SPME)

SPME is a sample preparation technique which is based on a concentration equilibrium between an extracting phase associated with a solid support and a biological matrix [115]. Two different SPME designs are the most commonly used: in-tube mode and fibre design. In this technique, small quantities of the sorbent are exposed to the headspace or solution of the biological sample using a suitable format. After a predefined amount of time and when equilibrium between the coating and the matrix has been reached, the sorbent does not absorb any additional quantity of the analyte of interest, meaning that the amount of drug extracted for a specific concentration is constant [116]. The main advantages of SPME are its low cost and the fact that it is time saving and environmentally friendly [117]. Some of its disadvantages are the slow time for equilibrium between the analyte and the extraction phase to be reached [118], the poor selectivity and the limited type of fibres commercially available [119].

Ultrafiltration

Ultrafiltration is a technique extensively used in food industry and water treatment. It is based on the selective passage of drugs of low molecular weight through the pores of a membrane of a specific MW cut-off, which inhibits the passage of molecules of higher MW. This technique does not involve time-consuming steps but its use is limited by reduced sensitivity due to interferences from the matrix in drug analysis and is usually used in combination with other clean-up techniques [89]. Ultrafiltration was successfully employed for the quantification of tetracyclines in milk, by adding solid EDTA at 10 mM concentration, before sample centrifugation at 5000 rpm for 1 h. Ultrafree MC/PL devices (nominal molecular weight limit 5000) were used in this study [120].

1.9. Conclusion

Prediction of gastric food-effect of drugs has been a challenge of the pharmaceutical industry. Even though the *in vivo* properties of the fed state gastric environment have been determined in some cases, the complicated fed environment due to differences in the meals administered makes the determination of the precise gastric conditions difficult. Despite the fact that some progress has been made with the development of gastric biorelevant media, a universal robust predictive analytical method has not been yet developed. The development of suitable biorelevant media in combination with a simple and robust analytical method could potentially provide a means of understanding of a potential food effect in regard of a drug's solubility and dissolution. Several biorelevant dissolution fed state media such as milk, nutrient

drinks or Fed State Simulated Gastric Fluid (FeSSGF) have been developed in an attempt to simulate the human postprandial conditions. Nevertheless, none have managed to achieve precise representation and fully overcome issues such as the need of a time-consuming preparation for the quantification of the drug, possible matrix interferences and compatibility with the analytical methods used.

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Chapter 2: Strategic drug analysis in fed-state gastric biorelevant media based on drug physicochemical properties

Abstract

Milk-based media such as the Fed State Simulated Gastric Fluid (FeSSGF) are commonly used in order to simulate the *in vivo* properties of the fed state stomach. Due to the lack of a specific guideline for standardised sample clean-up in these media, the aim of the current study was to develop an optimum protocol for the extraction and quantification of drugs from the fed state gastric medium based on the APIs' physicochemical properties (lipophilicity, ionisation, aqueous solubility and protein binding). Two different extraction techniques, protein precipitation (PP) and solid phase extraction (SPE) were assessed. A pilot study in six model drugs was performed, with tests using seven different protein precipitation reagents at four different medium:reagent ratios and two drug concentrations as well as different solid phase extraction cartridges and elution protocols. % recovery was analysed using partial least squares (PLS) regression so as to determine the physicochemical parameters affecting the drug percentage recovered. For protein precipitation protocols, drug concentration, selection of protein precipitation reagent and ratio added to the medium significantly affected drug % recovery from FeSSGF ($p < 0.05$). The same applied for the selection of elution solvent and cartridge type for solid phase extraction. Optimum protocols using MeOH, ACN and 10% w/v TCA at a 1:2 FeSSGF:reagent ratio were effective to a larger group of drugs of a wide range of lipophilicity and ionisation, with ACN being the most effective in the whole range of log P values (-0.56-8.81). Solid phase extraction was proven to be effective for compounds of poor to moderate lipophilicity (log $P < 4$), with extremely hydrophobic compounds demonstrating lower % recovery values (down to 10% recovery). PLS demonstrated that only for 10% w/v TCA (protein precipitation) and HLB (solid phase extraction) can the effect of key drug physicochemical properties on the final amount of drug recovered be accurately predicted.

Keywords: Fed state, Protein precipitation, Solid phase extraction, Biorelevant media, Drug analysis, Physicochemical properties

2.1. Introduction

The presence of food in the gastric environment as a factor affecting drug dissolution and absorption has been extensively discussed in the literature over the last 30 years. Even though most drugs are mainly absorbed in the small intestinal environment, the role of the gastric environment is equally important; the stomach acts as a reservoir, with the presence of food having a significant influence on the absorption of drugs through various mechanisms such as delayed gastric emptying [1], increased gastric residence time [2] and interaction of drugs with meal components [3].

The FDA proposes the use of high fat standard meals for the determination of a drug's food effect, as meals of high caloric and fat content can stimulate bigger changes in the gastrointestinal physiology and consequently have a more pronounced effect on drug bioavailability when this is affected by the presence/absence of food [4]. In theory, the optimal medium for the determination of food effect *in vitro* would be a homogenised standard meal, similar to the ones which have been successfully used for *in vivo* studies [5]. Due to difficulties in aspiration and handling of such a medium though [6], a range of biorelevant dissolution media has been developed in order to simulate the *in vivo* conditions of the fed state stomach. These media were developed with an aim of having the same physicochemical properties with the standard meal recommended by FDA for BA/BE studies [4].

Milk and milk-based media have been used as dissolution media for gastric fed state simulation for more than twenty years. Despite milk's simplicity and convenience though, its energy content differs to that of a standard high-fat breakfast used in BA/BE studies [7] and does not accurately simulate the gastric fed state conditions, because of deviations in osmolality and buffer capacity compared to the FDA-proposed standard meal [5]. Moreover, its pH value is significantly higher (pH ≈ 6.5) than the gastric pH after a meal administration (5.8 ± 0.2 at 50% of the meal emptied by the gastric compartment, liquid meal administered) [8]. In order to improve the gastric environment simulation, a milk-based medium called Fed State Simulated Gastric Fluid (FeSSGF) was developed. This medium consists of 3.5% fat milk, diluted with acetate buffer at 1:1 ratio [6, 9]. Three FeSSGF versions with different pH values have been used so as to mimic the three phases of gastric digestion with the pH values being 6.4, 5.0 and 3.0 for the early, middle and late phases respectively [10]. Despite its limited ability to simulate the gastric fed conditions at each point of ingestion, middle phase FeSSGF is used as a compromise for the reflection of the sum of gastric physiological events during ingestion

[10]. Unlike aqueous media, laborious techniques are usually required for extraction of drug from these milk-based media. A study where the extraction process was avoided [11] used an ion selective electrode (ISE) sensor system with two electrodes constantly in the dissolution vessel offering the possibility of a continuous dissolution profile. The method though was limited to the analysis of ionised drugs, it required a complicated correction of the baseline and was unable to analyse compounds of extremely low aqueous solubility.

Protein precipitation is the most frequently used sample clean-up technique, in which an organic reagent is added to the milk-based medium, followed by a centrifugation and a filtration step. Organic reagents like acetonitrile, added at 1:1 [12] and 1:2 [13, 14] FeSSGF:reagent ratios, isopropanol (1:1 ratio) [15] and ethyl acetate (1:4 ratio) [16] have been successfully used so far, but the rationale regarding the selection of the optimum reagent has not been yet clarified.

Solid phase extraction (SPE) is another extraction technique widely used for the extraction of drugs from heterogeneous media and biological fluids such as whole blood [17], plasma [18], urine [19], and milk [20], often preceded by a protein precipitation step. SPE cartridges consist of a polypropylene tube with the sorbent placed between two porous frits. Most cartridges are either comprised of bonded silica phases, similar to the material of the reversed phase HPLC columns but with bigger diameter particles (10-60 μm), or of polymeric resins (e.g. polystyrene-divinylbenzene) [21].

So far, there has been no specific guideline for the treatment of each compound according to its characteristics and every drug is examined separately as far as its effective extraction and quantification in fed gastric biorelevant media are concerned [22]. Due to the absence of a general *in vitro* predictive test, the aim of this study was the development of an optimised protocol for drug quantification in fed gastric biorelevant media, towards the buildup of an *in vitro* predictive test of food effect observed *in vivo*. To achieve the above, an analytical protocol in milk-based fed gastric biorelevant media dictating the optimum sample treatment maximising the method sensitivity was developed, providing an analytical roadmap guide according to the drug's physicochemical properties.

For the current study, a series of compounds of a wide range of lipophilicity and ionisation were selected as model compounds for the development of the analytical protocol, assessing the efficiency of the two extraction techniques mentioned above; protein precipitation and solid phase extraction. An extension of the multiple linear regression model (MLR), partial

least squares (PLS) regression was used to understand the impact of certain variables (drug lipophilicity, aqueous solubility, drug ionisation properties and protein binding) on the performance of two commonly used sample clean-up techniques for drugs dissolved in milk-based fed state biorelevant media. Its main advantage compared to the latter is its ability to analyse data with collinear independent variables [23]. To our knowledge, this is the first time the creation a general analytical guideline for a range of compounds in fed gastric media is being attempted. Moreover, an innovation of the study is the use of partial least squares regression in order to define the critical parameters which affect the efficacy of protein precipitation and solid phase extraction in fed gastric media, justifying their selection with statistical tools.

2.2. Materials and Methods

2.2.1. Materials

Furosemide (≥ 98% (HPLC), (±)-metoprolol (+)-tartrate salt (≥ 98% (titration)), 1,1-dimethylbiguanide hydrochloride (metformin hydrochloride, 97%), danazol (≥ 98%), itraconazole (≥ 98% (TLC)), celecoxib (≥ 98% (HPLC)), azithromycin (≥ 95% (NT)) and atovaquone (≥ 98% (HPLC)) were all purchased from Sigma-Aldrich, UK. Nifedipine (98 to 102% (on dried substance)), paracetamol (97.5% min. (HPLC)), atorvastatin calcium (pharmaceutical secondary standard; traceable to USP, PhEur), atenolol (≥ 98% (TLC)) and ketoconazole (inclusive between 98%) were all purchased from Fisher Scientific, UK. Pravastatin sodium (≥ 98%) and lapatinib (≥ 99% (HPLC)) were purchased from Carbosynth, UK. MK-C1, MK-C2, MK-C3 and MK-C4 were provided by Merck & Co, INC, US.

Sodium chloride, sodium hydroxide, sodium dodecyl sulphate, sodium acetate trihydrate, dipotassium hydrogen orthophosphate, potassium dihydrogen phosphate, ammonium acetate, hydrochloric acid 37% glacial acetic acid ≥ 99% and trichloroacetic acid 10% w/v were all purchased from Fisher Scientific, UK. HPLC grade methanol, ethanol, acetonitrile, acetone, trifluoroacetic acid (≥ 99%) were all purchased from Sigma- Aldrich, UK.

3.6% fat UHT milk was commercially purchased (Sainsbury's, UK).

Cronus 13 mm regenerated cellulose (RC) syringe filters 0.45 μm were purchased from LabHut Ltd, UK, Whatman 13 mm glass microfiber syringe filters 2.7 μm (GF/D) from Fisher Scientific, UK and SPE cartridges (Sep-Pak tC_{18} 3 cc Vac Cartridge, 500 mg Sorbent per Cartridge, 37-55 μm Particle Size, Sep-Pak C_8 3 cc Vac Cartridge, 500 mg Sorbent per

Cartridge, 37-55 μm Particle Size and Oasis HLB 1 cc Vac Cartridge, 30 mg Sorbent per Cartridge, 30 μm Particle Size) from Waters, UK.

2.2.2. Instrumentation

All samples were analysed in an HPLC system consisting of an Agilent 1200 series binary pump (G1312A), an Agilent 1200 series DAD detector (G1315D), an Agilent 1200 series autosampler (G1329A), an Agilent 1200 series controller (G1316A) and a Chemstation software (Agilent Technologies, Santa Clara, United States).

A pH meter Mettler-Toledo AG (model SevenCompact pH/Ion S220, Schwerzenbach, Switzerland), a centrifuge Hereus Biofuge Primo R (Thermo Scientific, Hanau, Germany), a vortex mixer Rotamixer (HTZ, Chesire, UK) and, a UV-Vis Thermo Spectronic Helios Gamma spectrophotometer (Thermo Scientic, UK) were used.

2.2.3. Fed state medium selection

Fed State Simulated Gastric fluid (FeSSGF) was selected as the working medium due to its simplicity in its preparation and stability for 72 h, with its pH, osmolality and buffer capacity remaining constant at ambient temperature [10]. Its buffer capacity, osmolality and surface tension values are in total closer to the values measured *in vivo* after the administration of a standard meal than the equivalent properties of milk, which has been extensively used as a gastric fed state medium in dissolution studies [24]. Finally, as it is less viscous than milk, its handing and loading/elution from the SPE cartridges was feasible without back pressure.

2.2.4. Medium preparation

Fed State Simulated Gastric Fluid (FeSSGF) was prepared according to Jantratid et al. [10], by mixing 3.6% fat milk and acetate buffer pH = 5 (17.12 mM CH_3COOH, 29.75 mM CH_3COONa, 237.02 mM NaCl in the medium) at a 1:1 volume ratio. For the preparation of 1 L of medium, 500 mL milk and 480 mL buffer were mixed under constant stirring using a magnetic stirrer. pH was adjusted to 5 with 1 N HCl and the volume was adjusted to 1 L with the buffer. Medium composition is described in Table 2.1.

Table 2.1. Fed State Simulated Gastric Fluid (FeSSGF) composition [10].

Sodium chloride (mM)	237.02
Acetic acid (mM)	17.12
Sodium acetate (mM)	29.75
Milk: buffer	1:1
HCl/ NaOH	qs pH 5
pH	5
Osmolality (mOsm kg^{-1})	400
Buffer capacity (mmol L^{-1} ΔpH^{-1})	25

2.1.5. Pilot study and selection of optimum conditions

20 drugs of a wide range of physicochemical properties (lipophilicity, ionisation, aqueous solubility and protein binding) were selected as model compounds (Table 2.2). Six compounds were selected for the pilot study in order to assess the optimum extraction techniques and protocols and determine the parameters affecting the extraction technique's efficiency. The rationale behind the selection of the compounds of the pilot study was the inclusion of active substances of a wide range of lipophilicity and ionisation properties, in order to assess their effect on the extraction method efficacy. The pilot study was performed on hydrophilic to extremely lipophilic (log P = -0.56–6.20) which included weak acids, weak bases and a neutral compound. Each compound's % absolute recovery was expressed as described in the equation (Eq. 1.1) below,

$$\%\ \text{absolute recovery} = \frac{\text{Area of peak of filtered aliquot}}{\text{Area of peak of standard solution of equivalent concentration in acetate buffer or MeOH: acetate buffer}} \quad \text{(Eq. 1.1)}$$

where filtered aliquot denotes the filtered drug solutions after protein precipitation or elution from the SPE cartridge.

In protein precipitation, four organic and three aqueous reagents were tested to determine the best % absolute recovery values; methanol (MeOH), ethanol (EtOH), acetonitrile (ACN) and acetone [organic reagents] and 2M HCl, 10% w/v trichloroacetic acid (TCA) and 10% w/v trifluoroacetic acid (TFA) [aqueous reagents]. Four different FeSSGF:precipitation reagent ratios (1:1, 1:2, 1:3 and 1:5) were tested to determine possible differences in % absolute recovery and two different concentrations (a "high and a "low", defined below Table 2.2) in

order to assess the method efficiency at a range of drug concentrations. The parameter assessed in SPE was elution volume, using tC_{18} cartridge (2 mL and 5 mL).

The efficiency of two extraction techniques [protein precipitation (PP) and solid phase extraction (SPE)] was investigated in the pilot study with the optimised protocol being applied to all compounds in Table 2.2. The optimum conditions (drug concentration, reagent and ratio) were selected and applied to all model compounds. The minimum efficiency limit for the pilot study was arbitrarily set to 50% (absolute recovery > 50%). Higher ratios (1:1, 1:2 were generally preferred due to higher method sensitivity (no need for dilution to overcome peak fronting). Similarly, for SPE, lower elution volume was preferred in case of similar recovery values, for higher method sensitivity.

Table 2.2. Physicochemical properties and working concentrations of model compounds.

Drug	log aqueous solubility (mg/ mL) [25-29]	log P [26, 28-41]	pKa [28, 39, 42-50]	Working concentrations (µg/ mL) (d)	Plasma protein bound (b)	BCS class
Metformin hydrochloride	2.48	-0.56	12.40	2000/2	0.035	III
Atenolol	1.11 (a)	0.23	9.60	200	0.129	III
Paracetamol	1.24 (a)	0.30	9.50	200	0.124	III
Furosemide	-1 (b)	0.74	3.90	80/1	0.031	IV
Metoprolol tartrate	1.01	1.95	9.70	200/10	0.244	I
Pravastatin sodium	-0.42 (b)	2.20	4.36	40	0.767	III
Nifedipine	-1.90	2.91	3.93	60/1	0.999	II
Propafenone hydrochloride	-0.82 (b)	3.39	9.27	600	0.957	II
Celecoxib	-2.52 (b)	3.47	11.10	100	0.975	II
Ketoconazole	-2.57 (a)	3.72	3.25, 6.22	150	0.986	II
MK-C1	-2.53 (c)	4 (c)	6.5 (c)	35	0.908	N/A
Azithromycin	3 (a)	4.02	8.74, 9.45	1000	0.558	II
Danazol	-3	4.20	none (b)	25/1	0.983	II
Atorvastatin calcium	-2.59 (a)	4.22	4.46	160	0.178	II

Atovaquone	-3.37	5.07	5.01 (b)	25	0.995	II
MK-C2	-3 (c)	5.11 (b)	4.48, 5.74 (c)	1300	0.985	N/A
Itraconazole	-6	6.20	3.70	0.5/0.1	0.997	II
Lapatinib	-5.68 (a)	6.30 (a)	6.34 (a)	8.7	0.998	IV
MK-C3	insoluble (c)	6.31 (b)	3.53 (c)	500	0.997	N/A
MK-C4	-4 (c)	8.81 (c)	none (c)	4.5	0.999	N/A

(a) Sci-Finder

(b) Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2016 ACD/Labs)

(c) Data provided by Merck and Co, INC

(d) "High" concentration/"Low" concentration:

"High" concentration = $\frac{drug\ dose\ (mg)}{500\ (mL)(volume\ typically\ used\ in\ gastric\ fed\ dissolution\ studies)}$ or solubility in mik/FeSSGF (literature values) or performed solubility study (24 h) in FeSSGF. "Low" concentration = 10 x LOQ in acetate buffer, MeOH:buffer (1:1) or ACN:buffer (1:1) and ≤ 0.2 x "high" concentration. Otherwise, 0.2 x "high" concentration was selected.

2.2.6. HPLC analysis

Stock solutions of the drugs were prepared in MeOH, EtOH, ACN or H_2O, based on the drug solubility in the above solvents. Calibration standards were prepared in organic solvent:"blank" acetate buffer 1:1 (pH adjusted to 5) mixture or acetate buffer pH 5, (where organic solvent is MeOH, EtOH or ACN, according to drug solubility in organic solvents). The drugs were analysed in HPLC with published HPLC methods (or modifications of published methods) which are stated in Table 2.3.

Adsorption studies were performed in triplicate for each model drug for all types of filters used. No adsorption issues were observed for the drugs studied.

Table 2.3. HPLC methods (or modification of published methods) used for the quantification of the model compounds.

Drug	Column	Mobile phase	Flow rate (mL/min)	Temperature (° C)	Inj. volume (µL)	UV detection (nm)
Nifedipine **[51]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 60:40	1	20	50	238
Furosemide **[52]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Formic acid 0.1% v/v 60:40	0.8	25	20	233
Metoprolol tartrate **[53]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:TFA 0.1% v/v 47:53	0.8	10	50	274
Danazol **[54]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 85:15	1	25	100	285
Metformin hydrochloride **[55]**	Vydac Diphenyl, 300Å, 250 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.02 M (pH = 7) 70:30	1	20	20	236
Itraconazole **[56]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 80:20	1	35	100	260
Celecoxib **[57]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 70:30	0.8	25	100	251

Atovaquone **[58]**	Waters Spherisorb S5 ODS2, C_{18}, 80Å, 250 x 4.6 mm, 5 µm	ACN:TFA 0.4% v/v 70:30	1.5	25	50	253
Paracetamol **[59]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 20:80	1	10	20	257
Ketoconazole **[60]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O:DEA 75:25:0.1	1	25	50	260
Atenolol **[61]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Phosphate buffer 0.01 M (pH = 4.5) 20:80	1	25	50	240
Azithromycin **[62]**	Waters Symmetry C_8, 100Å, 250 x 4.6 mm, 5 µm	MeOH:Phosphate buffer 0.3 M (pH = 7.5) 20:80	1.2	40	100	210
Pravastatin sodium **[63]**	Agilent Eclipse XDB C_{18}, 120Å, 250 x 4.6 mm, 5 µm	MeOH:Phosphate buffer 0.03 M (pH = 7) 55:45	1	25	100	238
Lapatinib **[64]**	Agilent EC-C_{18} Poroshell, 150 x 4.6 mm, 2.7 µm	ACN:Ammonium acetate 0.05 M (pH = 4.5) Gradient (0-5 min 40:60/ 5-13 min 58:42/ 13-17 min 90:10/ 17-19 min 40:60)	0.9	40	50	261

Propafenone hydrochloride **[65]**	Agilent Eclipse XDB C_{18}, 120Å, 250 x 4.6 mm, 5 µm	MeOH:ACN:TEA: H_2O 50:7.5:0.1: q.s 100 (pH= 2.9)	0.8	25	20	248
Atorvastatin calcium **[66]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.025 M (pH = 6) 40:60	1.5	30	50	246
MK-C1*	Waters Symmetry Shield C_{18}, 100Å, 50 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.025 M (pH = 2.5) Gradient (0-2 min 65:35/ 2-2.01 min 90:10/ 2.01-3 min 90:10/ 3-3.01 min 65:35)	3	40	20	214
MK-C2*	Phenomenex Onyx monolithic $C_{18,}$ 300Å, 100 x 4.6 mm	ACN: 0.1% H_3PO_4 70:30	5	40	10	240
MK-C3*	Agilent Prorochell C_{18}, 120 Å, 50 x 2.7 mm	ACN:Sodium Phosphate 0.005 M (pH = 7) Gradient (0-0.5 min 40:60/ 3-3.5 min 10:90/ 3.51-5 min 40:60	1	40	25	250
MK-C4*	Phenomenex Onyx monolithic $C_{18,}$ 300Å, 100 x 4.6 mm	ACN:H_2O 70:30	3.5	40	100	220

*HPLC methods were provided by Merck and Co, INC

2.2.7. FeSSGF solubility studies

Where FeSSGF or milk solubility data was not available in the literature, drug 24 h-solubility values in FeSSGF were determined by using a modification of a protocol used by Wagner et al. in FeSSGF [15]. The solubility of the model compounds was determined by weighing excess amounts of the drug into 5 mL Eppendorf tubes, followed by the addition of 5 mL of FeSSGF. The samples were left to equilibrate in a shaking water bath at 37 °C for 24 hours, and then filtered through a GF/D filter of 2.7 μm pore size. 1 mL of ACN was added to 0.5 mL of the filtered sample, vortexed for 30 sec and centrifuged (15 min, 8000 rpm, 4 °C). The supernatant, was filtered through a 0.45 μm RC filter, diluted and analysed using HPLC. Drug was quantified against calibration standards in FeSSGF which had undergone the same treatment as the sample. Each measurement was performed in triplicate.

2.2.8. Protein precipitation (PP)

2.2.8.1. Protein precipitation methodology

1 mL of working solution of each drug in FeSSGF was placed in a plastic centrifuge tube. A volume of the protein precipitation reagent according to the FeSSGF:protein precipitation reagent ratios as defined below (1, 2, 3, 5 mL) was added. The mixture was vortexed at full speed for 30 sec and centrifuged at 8000 rpm (9800 × g) for 15 minutes (4 °C). The supernatant was filtered through a 0.45 mm RC filter and assayed. The sample was diluted with acetate buffer or MeOH:acetate buffer 1:1 when diluent was more highly eluting than the mobile phase and peak shape needed to be improved.

2.2.8.2. Drug purity in the supernatant

Full scans of the supernatants of the six drugs used in the pilot study plus atovaquone (a compound which demonstrated big differences in recovery between the three optimum reagents used.), with MeOH, acetonitrile and 10% w/v TCA used as protein precipitation reagents, were performed using the diode array detector of the HPLC instrument. Scans were performed over a range from 190 to 400 nm. Standards were prepared in a mixture comprising one part of buffer and two parts of PP reagent so as to maintain the same amount of precipitation reagent as the extracted FeSSGF samples with the selected reagents. Spectra of a supernatant after proteins were precipitated with a specific reagent and spectra of the same drug, dissolved in 1 part of acetate buffer pH = 5 and 2 parts of the selected protein precipitation reagent were

normalised to peak intensity and superimposed using the "best possible match of the entire spectrum" mode in Chemstation software.

2.2.9. Solid phase extraction (SPE)

Three different types of cartridges were used: tC_{18} (500 mg bed weight), HLB (30 mg bed weight), C_8 [(500 mg bed weight)-used for the extraction of metformin hydrochloride only]. The extraction cartridges were conditioned by washing with 5 mL [tC_{18} (trifunctional octadecyl silica), C_8] or 1 mL MeOH (HLB), followed by 5 mL and 1 mL of H_2O respectively. 1 mL of FeSSGF was loaded and the columns were 5 washed with 5 mL and 1 mL of H_2O respectively. The drugs were eluted with 5 or 2 mL MeOH:H_2O 70:30 (tC_{18} and C_8 cartridges) or 1 mL MeOH (HLB cartridges). The main difference between tC18 and classic C_{18} cartridges is the ability of the former to be used in extreme pH values (pH < 4 and pH > 7).

As in the case of protein precipitation, a pilot study with the initial six compounds was performed and the optimal conditions of the parameters examined were applied for the rest of the model compounds. Modifications of the above protocols were performed in cases of % absolute recovery values < 50%, with different approaches according to each drug's physicochemical properties and are described in detail in SPE protocol optimisation part of Results and Discussion section. Protocols were optimised by: ***modifications in cartridge conditioning*** (a. use of an ion-pair reagent, b. conditioning of the cartridge with an acid or a base so as to improve its retention characteristics) or ***modifications in elution*** (a. use of different elution solvents, b. addition of acid or base in elution solvent so as to increase its elution strength). Specifically:

Metformin: A C_8 cartridge which retains hydrophilic compounds better was used and either the washing step was omitted or the HLB cartridge was pre-treated with 2 mM Sodium dodecyl sulphate (SDS) solution before the loading step. SDS was selected based on the hypothesis that due to the stationary phase's chemistry, the equilibration of the HLB cartridges with an ion pair reagent would lead to the retention of the drug to the cartridge through development of hydrophobic interactions between drug and cartridge with the complex easily be broken during the elution of the drug with an organic elution solvent [67]. *Atovaquone, lapatinib, MK-C1, MK-C2, MK-C3, MK-C4*: Elution with MeOH for the more effective disruption lipophilic interactions between the drug and the lipophilic chains of the tC_{18} cartridge. Itraconazole: Pre- treatment of the cartridge with 0.01 M NaOH (to retain the drug

(weak base) on the cartridge (HLB) and elution with 0.25 M formic acid in MeOH for a more efficient elution in its ionised form.

2.2.10. Statistical Analysis

Comparisons were performed in order to assess significant changes in drug recovery using different precipitation reagents, medium:reagent ratios, drug concentration, different SPE cartridges and elution volumes. For protein precipitation, % absolute recovery and correlation with added protein precipitation reagent, (FeSSGF:reagent ratio) and drug concentration were evaluated in the context of a multiple way Analysis of Variance (ANOVA) (Statgraphics v. XVI, StatPoint Technologies Inc, US) with a post-hoc Bonferroni test. In solid phase extraction, effect of different elution volumes and cartridges on drug % absolute recovery were compared using a two-tailed t-test. (Statgraphics v. XVI). Comparisons where $p < 0.05$ suggested a statistically significant difference.

The absolute % drug recovery using different protein precipitation or SPE protocols was correlated to drug physicochemical properties by partial least squares (PLS) regression using the XLSTAT software (Microsoft, US). The parameters evaluated were: lipophilicity (log P), log aqueous solubility in mg/mL, drug unionised fraction at pH = 5, acid/base properties and drug protein bound fraction in plasma proteins (drug bound fraction to plasma proteins was used as a measure of protein affinity due to the lack of available data in milk proteins in the literature). The physicochemical properties selected as independent variables were decided on the basis of their potential effect on drug distribution in the aqueous and lipid phases of the medium and its interaction with milk proteins. PLS regression analysis was performed with % recovery of the three reagents used for the extraction of the 20 model compounds being the dependent variable. Selected interactions were also included in the model (log P*log aqueous solubility, log P*acid/base properties, aqueous solubility*drug unionised fraction, aqueous solubility*acid/base properties, unionised fraction*acid/base properties). The model quality was evaluated on the square of the coefficient of determination (R^2) and goodness of prediction (Q^2). R^2 and Q^2 values close to 1 refer to a model of good fit and prediction power respectively while a difference lower than 0.2-0.3 between them is indicative of a successful model [68]. Full cross-validation (leave-one-out procedure) was used to develop and evaluate the regression model. The optimum number of calibration factors for each model was selected based on the model's optimum predictability (Q^2) and predicted residual error sum of squares (PRESS). A Q^2 value > 0.5 is generally considered acceptable for good model

predictability [69]. Lower PRESS values indicate better prediction [70] with the number of latent variables where PRESS starts increasing indicating the number of variables which to be retained in the model [71]. The standardised coefficients of the factors plotted indicate the relative positive/negative effect of their corresponding variables on the % drug recovery (response value). High standardised coefficients for variance X have a big positive or negative effect on response Y. The importance of each parameter was evaluated by its variable importance in projection (VIP) value. Values above 1.0 are considered to have a significant effect on the dependent variable, whereas values < 0.7-0.8 are not of significance for the prediction of the dependent variable [68].

2.2.11. Roadmap design

The roadmaps leading to selection of optimal protein PP and SPE protocols for drug analysis were constructed combining the results from the complete study for the 20 model drugs (and selected PP and SPE conditions) and the variables affecting the drug percentage recovered, as demonstrated by the PLS regression analysis. Only models with Q^2 values > 0.5 were considered for the roadmap design. For protein precipitation, optimum conditions were selected on the basis of absolute % absolute recovery. If absolute % recovery was > 85% for more than one reagent, MeOH or 10% w/v TCA were preferred over ACN, as they give peaks of better shape without the need of dilution.

2.3. Results and Discussion

2.3.1. Drug analysis: Optimisation of protein precipitation conditions

2.3.1.1. Pilot study and selection of optimum extraction conditions

When added to media containing proteins, organic reagents act by decreasing the dielectric constant of the proteins of the medium, a. increasing electrostatic interactions between them and b. displacing water molecules around their hydrophobic areas. Thus, their solubility in the medium decreases, leading to aggregation and protein precipitation [21].

All four organic reagents used (MeOH, EtOH, ACN, acetone) gave acceptable recovery values (> 69.5%) for the six model compounds in the pilot study with clear supernatants for drugs' analysis in the HPLC after filtration (Figure 2.1). The only exception was EtOH when was as a precipitation reagent for danazol (Figure 2.1), which resulted in poor peak shape in HPLC despite the dilutions made.

Acidic reagents act by forming insoluble salts with the positively charged amino acids of a milk-based medium at pH below their isoelectric point [21]. The use of weak acids as protein precipitation reagents may be challenging for drugs demonstrating instability in acidic conditions. Hydrochloric acid, trichloroacetic acid and trifluoroacetic acid were particularly effective as precipitation reagents giving high recovery values (92.4–106.7%) for all ratios of hydrophilic (metformin, metoprolol) (Figure 2.1). They were not able to recover high amounts of the two most lipophilic drugs (danazol, itraconazole) from the medium though, with 2.4 ± 0.1% maximum recovery at a 1:5 ratio for danazol and 0% recovery for all ratios for itraconazole achieved. As expected, a reason for the poor recoveries of lipophilic compounds in acidic reagents is their lower aqueous solubility, which is a barrier for the extraction potential of compounds of similar lipophilicity. The two weak acids (nifedipine, furosemide) were partially recovered using acidic reagents with the recovery percentage ameliorating by decreasing the FeSSGF:reagent ratio. The two weak bases (metformin, metoprolol tartrate) were almost 100% recovered at all ratios (Figure 2.1).

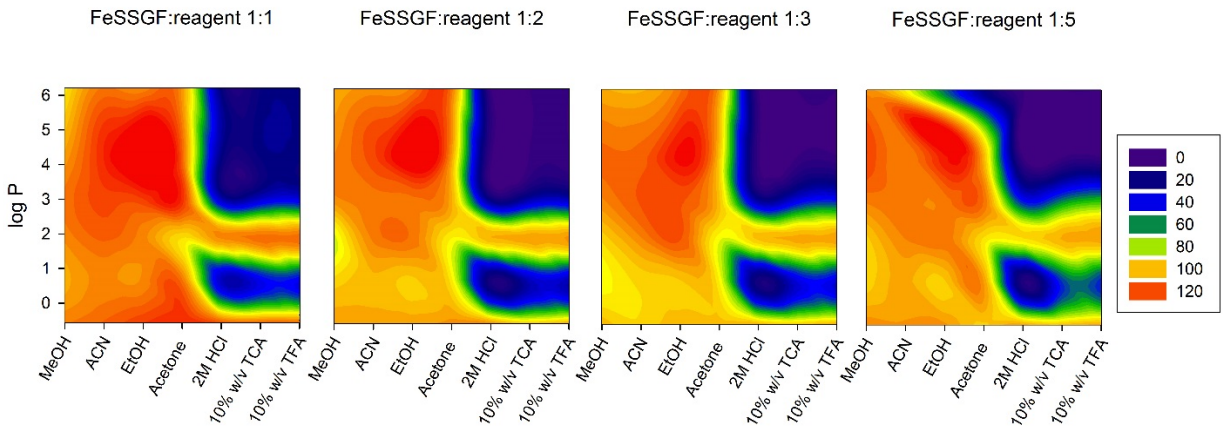


Figure 2.1. Protein precipitation reagent-ratio-log P gradient map; contour plot of % recovery after protein precipitation of drug solution at "high" concentration in FeSSGF for the six compounds of the pilot study. "Warm" colours (red, orange) indicate high recovery values and "cold" colours (green, blue) indicate poor reagent performance.

A three-way analysis of variance showed that the selection of protein precipitation reagent affected the % recovery values for all six drugs of the pilot study ($p < 0.05$) (Figure 2.2). Acetonitrile was proven the most effective (higher mean absolute recovery) for three out of six drugs, acetone for two and ethanol for one compound respectively in terms of mean absolute recovery. The presence of NaCl in the medium can increase drug recovery when acetone is used as protein precipitation reagent. Crowell et al. [72] demonstrated that in acetone concentrations between 50 and 80% of the total mixture, NaCl concentrations > 10 mM in the

medium led to protein % recovery values close to 100% for a number of proteins, such as α-casein, β-lactoglubulin and bovine serum albumin which are present in milk [73]. The effective entrapment of proteins in the precipitate possibly led to an increased amount of free drug available in the supernatant, resulting in higher recovery. Differences in efficiency among precipitation reagents can be attributed to the remaining proteins in the supernatant; since protein precipitation can only remove the larger proteins, leaving small proteins and peptides behind. These may interfere with the compounds of interest and have unpredictable effects (such as unexpectedly low drug recoveries) on drug quantification [21].

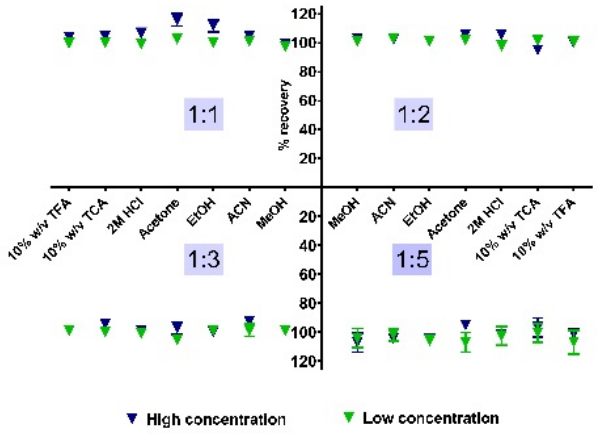
The effect of the FeSSGF:precipitation reagent ratio was evaluated in the pilot study for achieving maximum absolute drug recovery and adequate method sensitivity. Decreasing the medium:precipitation reagent ratio (from 1:1 to 1:5) did not show profound differences in drug absolute recovery as far as organic reagents were concerned. With the exception of itraconazole, for which the % absolute recovery increased from approximately 78% to 99.9-106.6% when decreasing the FeSSGF:organic reagent ratio from 1:1 to 1:2, 1:3 and 1:5, all organic reagents resulted in % absolute recovery > 80% at all ratios used (Figure 2.2). A higher amount of organic solvent may increase the percentage of drug recovered by reducing solvation of the proteins in the aqueous medium, causing their precipitation. The statistical analysis showed that the ratio in which the precipitation reagent was added was statistically important. Reported p values for 4/6 drugs used in the pilot study were < 0.05 with the recoveries of metoprolol tartrate and danazol not being affected ($p = 0.86$ and 0.66 respectively) by the amount of precipitation reagent added (Figure 2.2). For the other drugs, 3 or 5 parts of protein precipitation reagent added in 1 part of FeSSGF resulted in higher % drug recovery than 1 part of reagent added to 1 part of FeSSGF prior to vortexing and centrifugation (Figure 2.1; red parts of the contour plot). Even though the differences among the protocols with different ratios were statistically significant, the difference may not always be practically important, as in most cases the method efficiency threshold set for the study (50% absolute recovery) was met. Nifedipine is given as an example; the addition of 1 part of methanol in 1 part of FeSSGF, recovered approximately 101% of the drug, while addition of 5 parts recovered approximately 107% (Figures 2.1, 2.2). The same protocol by using 10% w/v TFA gave 26% and 72% values respectively. It is obvious that in the first case selection of a 1:5 ratio would not improve the extraction method but it would result in a loss of sensitivity, due to the bigger dilution of the medium with methanol. In the second case though, the difference is notably important and

therefore for a compromise, % recovery, desired method sensitivity and HPLC method compatibility with the medium have to be considered.

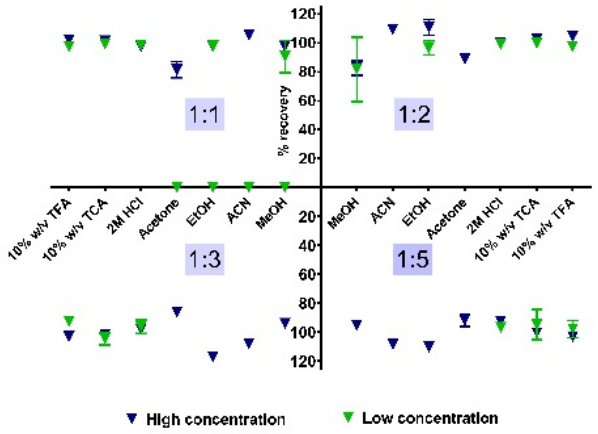
Drug concentration had an effect on the percentage recovered using organic or aqueous solvents for protein precipitation. For the hydrophilic base (metformin), the % recovery values were not affected by the drug concentration ($p > 0.05$) (Figure 2.2). In all other drugs of the pilot study, drug at high concentration was more effectively recovered ($p < 0.05$). Similarly to the example given above for the effect of protein precipitation ratio, the average difference between recoveries of "high" and "low" concentrations as given by the post-hoc Bonferroni test lied within a range between 0.5 and 12.3% in the range of drugs studied, with the highest recovery observed for "high" concentrations. Despite the slight differences in absolute recovery between concentrations, the method can still be used for drug analysis if the 50% absolute recovery limit is met and linearity is proven in the working concentration range. Reagents added at a 1:3 or 1:5 ratios to FeSSGF often dilute the sample significantly, driving its recovery below the LOQ of the method for the "low" concentration (Figure 2.2).

The reagents (two organic and one aqueous) selected were methanol, acetonitrile and 10% w/v trichloroacetic acid at a 1:2 FeSSGF:reagent ratio with the rationale of selection explained in the Materials and Methods part. Since the pilot study proved that the volume of reagent added for protein precipitation was statistically important, a relatively high reagent: FeSSGF ratio was selected (1:2), so that high % recovery and adequate method sensitivity could be maintained. For the final study and assessment for the rest of the model compounds, the higher of the two concentrations, which gave higher % recovery values in the pilot study was chosen.

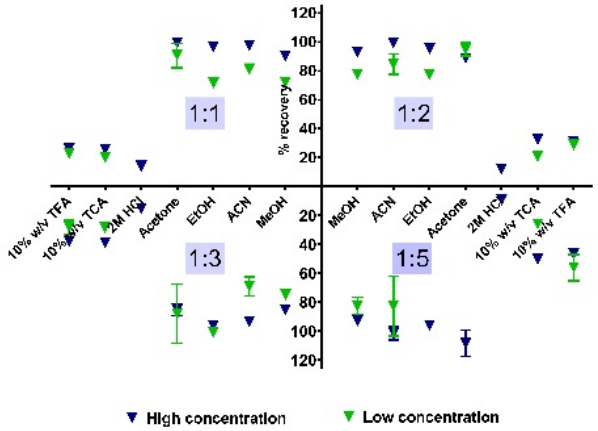
	p concentration	p reagent	p ratio
Metformin hydrochloride	0.407	***	***
	***	***	***



	p concentration	p reagent	p ratio
Metoprolol tartrate	*	***	0.861
	***	***	***

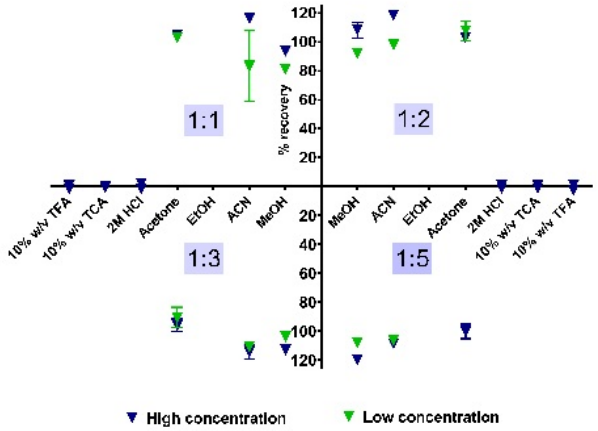


Furosemide

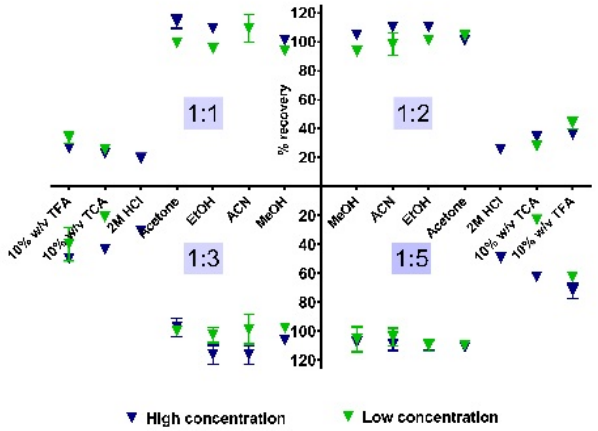


***	***	0.663

Danazol

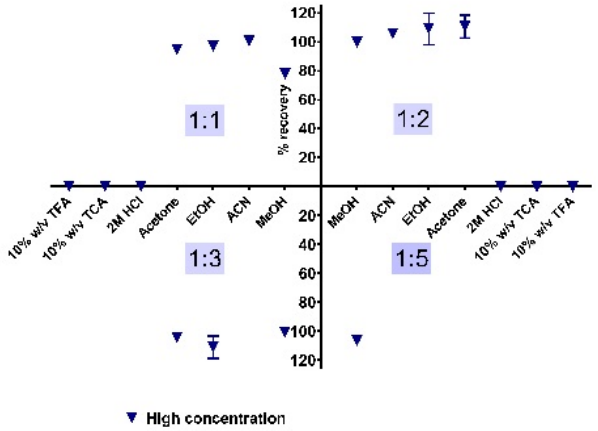


Nifedipine



***	***	-

Itraconazole§



§ two-way analysis of variance (ANOVA) performed for itraconazole due to the inability of the method to detect the drug at "low" concentration.

Figure 2.2. Three-way ANOVA results of protein precipitation conditions for the six drugs of the pilot study. Graphs denote % drug recovery for all reagents at high (blue) and low (green) concentrations. Purple squares denote the FeSSGF:reagent ratio used in the protocol (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Bonferroni post-hoc test).

2.3.1.2. Main study and selected protocol application

The two organic reagents used, methanol and acetonitrile, added at a 1:2 FeSSGF:reagent ratio, gave high recovery values for all the compounds of a wide range of lipophilicity (log P = -0.56-8.81) and ionisation with the exceptions of atovaquone and MK-C4. Atovaquone was only recovered by 33.4% at 25 μg/mL in FeSSGF when methanol was used, while acetonitrile recovered 82.2% of the same drug concentration (Figure 2.3). For extraction with acetonitrile, atovaquone studies in in plasma [74] and whole blood [75] gave similar results to our study. For methanol, since atovaquone's solubility in it is much higher than the concentration used, a possible reason of the significantly low recovery values could be the loss of analyte due to its occlusion in the precipitate [76]. The drug's extremely high affinity for plasma proteins (> 99.5% bound) [77] and its high affinity to fat, as described in *in vivo* studies which showed increased drug bioavailability after co-administration with a high fat meal [78], could indicate a strong interaction with fat or proteins of the fed state medium. This interaction may have not been disrupted by the application of methanol, with the drug being entrapped in the precipitate.

The aqueous reagent (10% w/v TCA) added at the ratio mentioned (1:2) was proven effective only for highly soluble (aqueous solubility > 100 μg/mL) APIs with log P values < 2 with the % recovery of weak bases (metformin hydrochloride, metoprolol tartrate, atenolol, paracetamol), approaching 100% (Figure 2.3). The above compounds were mostly unionised at pH 5 but were likely negatively ionised at the low pH of the acidic supernatant. Precipitation with trichloroacetic acid gave poor recovery values (0%) for atovaquone, because the drug as a lipophilic weak acid co- precipitated with the proteins [79]. The same applied for MK-C4, which is an extremely lipophilic drug with high affinity for adipose tissue [80].

% recovery values lower than 100% when organic reagents were used for protein precipitation were probably not attributable to the interaction of the drug with protein molecules of the supernatant, but to its entrapment in the precipitate. This hypothesis was confirmed by superimposing the spectra of the supernatant and drug standards in an acetate buffer:precipitation reagent mixture. The drugs selected demonstrated variable % recovery values in the three reagents selected (methanol, acetonitrile, 10% w/v TCA). Nevertheless, the spectra were identical in all cases despite high or low % drug recovery values and no other interference was observed in the peak of the drug. Moreover, the retention time of all drugs in the chromatogram remained constant, which implied that there was no change in the structure

of the drug molecule (data not shown). Therefore, the results indicated that the drug quantified was the free drug in solution without any interference from the biological matrix.

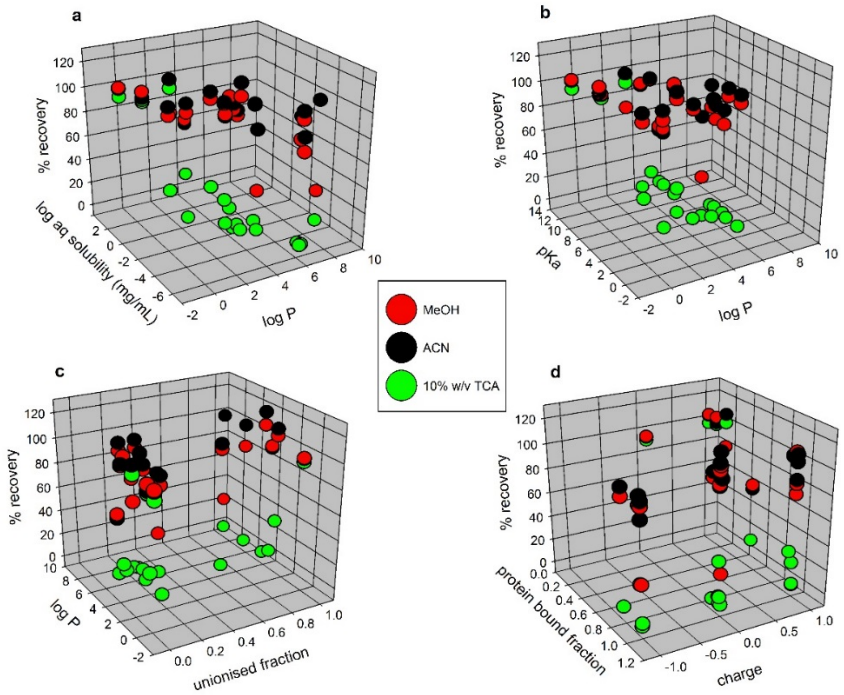


Figure 2.3. Mean % recovery values of the selected protein precipitation reagents (MeOH, ACN, 10% w/v TCA) for the 20 model drugs (Table 1), **a.** vs. log P and log of aqueous solubility in mg/mL, **b.** vs. log P and pKa, **c.** vs unionized fraction and log P and **d.** vs charge vs protein bound fraction as 3D scatter plots.

2.3.1.3. Prediction of the effect of physicochemical properties on extraction protocol (PP) selection

The variables and their interactions of the PLS models examined are summarized in Figure 2.4. The PLS models constructed for % recovery values when MeOH, ACN and 10% w/v TCA were used as protein precipitation reagents were defined by 1, 1 and 2 Principal Components respectively. The PLS model developed for 10% w/v TCA was a good fit to the experimental values ($R^2 = 0.87$) and showed good predictive power ($Q^2 = 0.83$). The models developed for MeOH and ACN can only account for a very low percent of Y variability ($R^2 = 0.34$ and 0.23 respectively), and have limited predictive power ($Q^2 = 0.24$ and 0.05 respectively), according to the threshold ($Q^2 = 0.5$) set for the study.

The model demonstrated that lipophilicity (log P) is defined as negative predictor for % drug recovery in all three cases when MeOH, ACN and 10% w/v TCA were used as protein precipitation reagents in FeSSGF treatment with its effect in the cases of MeOH and TCA 10%

w/v being statistically significant, as indicated by the high VIP factor (Figure 2.4). A higher partition coefficient indicates higher tendency of the solutes distribution to the lipid phase of the medium [81], which could be limiting the extraction potential of the reagent.

In cases where methanol was selected as protein precipitation solvent, drug lipophilicity affected the extraction of compounds regardless of their ionisation state, with a bigger effect on neutral and acidic compounds (VIP > 1), and compounds being in the unionised state in the working pH, as indicated by the negative standardised coefficients of log P interactions with the properties mentioned. Drug distribution in the lipid medium fraction is facilitated for unionised drugs, as ionised molecules have to dispose a part of their hydration water in order to permeate the lipid bilayer, a process energetically unfavourable [82]. The above observations of the drug physicochemical properties which affect extraction from FeSSGF using methanol, denote that even though the effect of ionisation percentage does not have a significant impact by itself, it can have a negative effect of the amount recovered in lipophilic drugs.

For the use of 10% w/v TCA in protein precipitation, the main factors governing the % recovery are the drug's log P, drug bound protein fraction (negative effect) and its aqueous solubility (positive effect). Even though the exact mechanism of protein precipitation is not fully understood, a proposed mechanism of action suggests the segregation of the protein bound water, with the type of the proteins not affecting the method efficiency, which is also acid-concentration dependent [83]. The concentration of TCA used in the current study may only be adequate to precipitate a portion of proteins, with the drugs highly bound to proteins being trapped in the precipitate. High lipophilicity and high aqueous solubility as negative and positive predictors respectively may be explained by the aqueous nature of the precipitation reagent. Moreover, the fact that the interactions of drug protein bound fraction with log P and aqueous solubility have a negative effect on drug recovery, strengthens our hypothesis that the inability of the TCA to break the drug-protein interactions under the stated experimental conditions is an unfavourable factor, even for the extraction of water soluble drugs. As observed in the pilot study, PLS regression showed that TCA is suitable reagent for highly soluble weak bases (positive log aq sol*base interaction, Figure 2.4), while it affects the extraction of lipophilic bases or bases which are unionised in the medium's pH in a negative manner, which was demonstrated by the negative log P*base and union fr*base negative standardized coefficients (Figure 2.4).

The lack of the model's predictive ability when ACN was used as a PP reagent can be explained by the reagent's extremely high extraction ability in the whole range of drugs (> 90% recovery for 17/20 drugs studied) and low variance, with most values falling close to 100%.

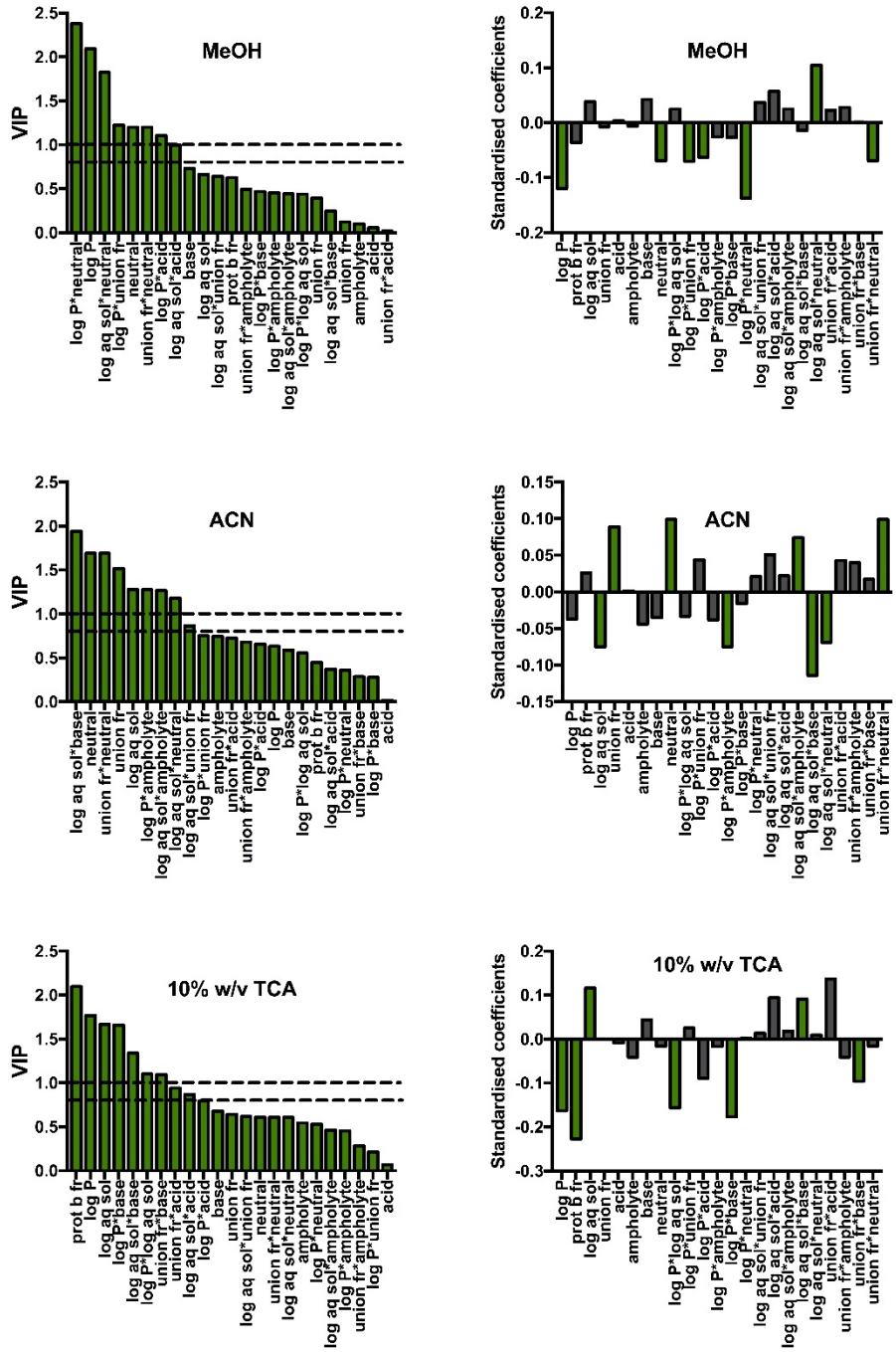


Figure 2.4. Variable importance in the projection (VIP) plot with the variables classed according to their importance of the response (left) for the selected protein precipitation protocols. Standardised coefficients corresponding to the variables (and their interactions) studied. Green colour denotes coefficients of VIP values > 1, which are considered influential to the response value (right).

2.3.1.4. Designing a roadmap for effective sample treatment using protein precipitation

In summary, MeOH and ACN can be effective for drugs of a wide range of lipophilicity and the use of one of the other is usually effective for drugs of log P values of -0.5 to 5 (Figure 5). For hydrophilic to moderately lipophilic bases (metformin, metroprolol tartrate, log P < 2), the use of 10% w/v TCA was preferred over the two organic solvents, due to a better peak shape in the HPLC analysis. According to the findings of the PLS regression analysis, TCA is the most efficient reagent (higher absolute % recovery) for highly soluble drugs and drugs which exhibit a low affinity for proteins. Drugs of moderate lipophilicity were equally well recovered using either MeOH or ACN, therefore, both reagents could be used for the extraction of drugs of log P between 2 and 5. Issues with low % recovery with the use of MeOH were only encountered some drugs of extreme lipophilicity (log P > 5), for which ACN was the most effective option (atovaquone, MK-C4). Consequently, ACN would be the best choice of the three reagents (Figure 2.5), as drugs at this log P range were in all cases at more than 80% which could give absolute recoveries as low as 24 and 0% respectively.

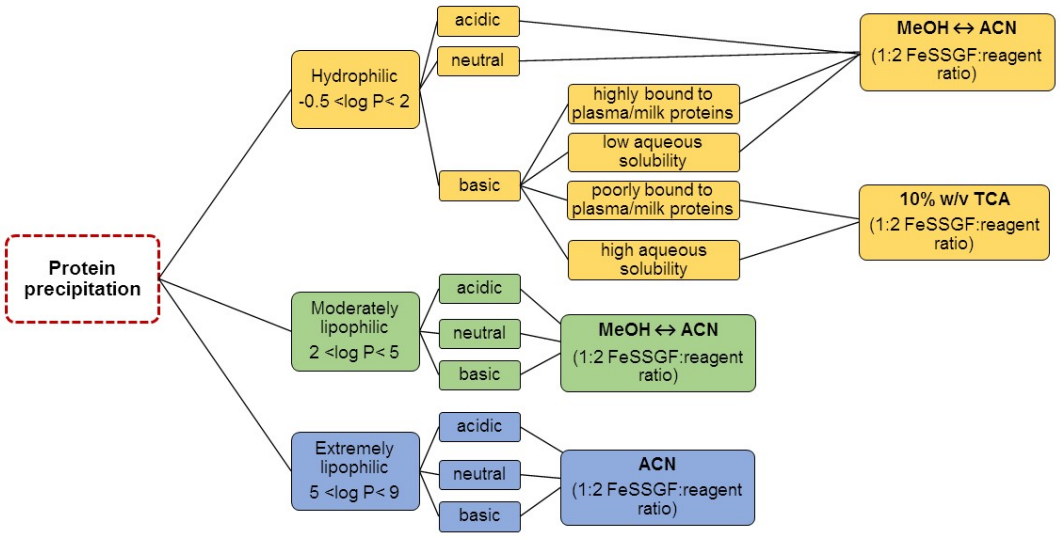


Figure 2.5. Roadmap of protein precipitation conditions selected for maximum % drug recovery from the fed gastric medium.

2.3.2. Drug analysis: Optimisation of solid phase extraction conditions

2.3.2.1. Pilot study and effect of elution volume

The results of the pilot study, where tC_{18} cartridge was selected as a starting point are presented in Figure 2.6b. It can be seen that the specific cartridge can be effectively used for a range of compounds from moderately polar to non-polar. Drugs of log P between 0.74 and 4.2 were recovered at a percentage higher than 60% (Figure 2.6b). Even though the minimum elution solvent (2 mL) is higher than two bed volumes (500 mg sorbent = 600 μL bed volume) which is required for effective extraction [21], it was shown that elution with 5 mL increased the % recovery values by a significant amount ($p < 0.05$) compared to 2 mL for all compounds of the pilot study (from 6.5% increase for nifedipine to 1700 % increase for danazol) (Figure 2.6b). Therefore, 5 mL were selected as the elution volume to proceed with the rest of the model compounds.

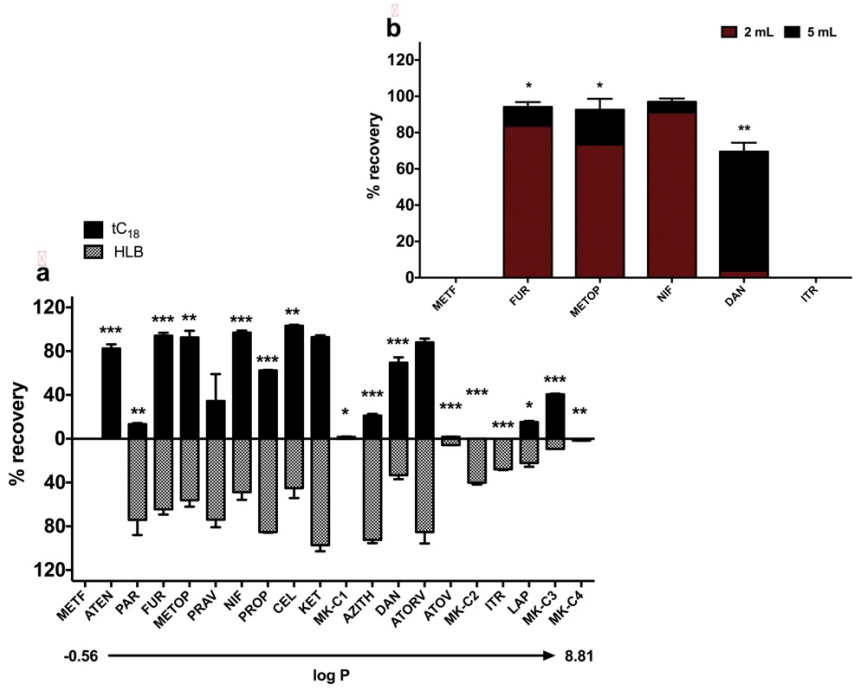


Figure 2.6. a. % recovery values of model drugs using the SPE protocols for tC_{18} and HLB cartridges. **b.** % recovery values of model drugs of pilot study using different elution volumes (tC_{18} SPE cartridge). Stars denote significant differences between % recoveries of **a.** different cartridges and **b.** elution volumes (b) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided t-test).

2.3.2.2. Effect of cartridge

The results of the complete study where the cartridges and elution conditions selected were applied for all of the model compounds are presented in Figure 2.6a. tC_{18} cartridges can

generally be used for a range of compounds from moderately polar to non-polar. Non-polar parts of the analyte develop Van der Waals interactions with the C_{18} non-polar groups of the sorbent, leading to selective retention of the analyte of interest, before its elution with an appropriate elution solvent. HLB was another type of sorbent used in this study and is a co-polymer comprised of two different monomers; one hydrophilic (N-vinylpyrrolidone) and one hydrophobic (divinylbenzene). The use of HLB cartridges has also been found to be effective for both polar and non-polar compounds [84].

For compounds of log P values between 1.95 and 4, there was no clear pattern as to which should be selected in favour of the other for optimum % recovery values using log P as a selection criterion. Nevertheless, the use of one or the other cartridge (tC_{18} or HLB) in compounds of moderate lipophilicity (log P 2-4) recovered a minimum value of 69.5% of the initial compounds in FeSSGF for the optimum protocol at each case (Figure 2.6a). MK-C1, a compound on the verge of the threshold set for moderate lipophilicity (log P = 4) was poorly recovered in all cases 1.76 ± 0.33% and 0.15 ± 0.0 %for tC_{18} and HLB cartridges respectively. Its recovery was not improved despite protocol modification (tC_{18} and elution with MeOH, recovery = 8.80 ± 0.61).

For most hydrophilic and lipophilic compounds, HLB was proven more effective, as it increased the % recovery values of the compounds which could not be effectively extracted (< 15% absolute recovery) using tC_{18} cartridges (metformin hydrochloride, paracetamol, atovaquone, itraconazole, MK-C1, MK-C2, MK-C4), but not always to a great extent. For the drugs which could not be effectively extracted with the protocol used with tC_{18} cartridges, a switch to HLB achieved a meaningful improvement in extraction performance only for paracetamol MK-C2 (Figure 2.6a).

SPE was incompatible with the extremely lipophilic model drugs studied. Using the current protocols, the recoveries of drugs of extreme lipophilicity (log P > 5) were higher with the use of HLB cartridges but still relatively low (5.7 ± 0.2%, 27.9 ± 0.7%, 22.1 ± 3.5% and 0% against 1.9 ± 0.1%, 0 %, 15.3 ± 0.2% and 0% for tC_{18}, for atovaquone, itraconazole, lapatinib, and MK-C4 respectively (Figure 2.6a). Previous studies with extraction of itraconazole with HLB cartridges from biological matrices demonstrated higher recovery values than the ones presented in this study. Although HLB cartridges have been more successfully used for extraction of itraconazole, these studies were in blood, [85] plasma [86] and surface waters [87] and the cartridges could be incompatible with the fed state medium

used. The modification proposed in the methodology did not improve the recovery of the drug (Figure 2.7). The low recoveries of the extremely lipophilic compounds and the lack of pattern in terms of cartridge selection for the extraction of the moderately lipophilic compounds could indicate that the critical parameter for SPE optimisation is not the log P value of an API, but the type of interactions it develops with components (lipids, proteins) of the milk-based matrix. For atovaquone, 5 mL MeOH were also tested with tC_{18} cartridge giving somewhat better results but still low recovery values (17.5 ± 0.6 %) (Figure 2.7). The poor SPE recovery values for the specific drug, along with the low % recovery when MeOH was used in protein precipitation, supports the initial hypothesis that strong interaction with components of the fed state medium could be the main obstacle which has to be surpassed for effective extraction. In the cases of lapatinib, MK-C2, MK-C3 and MK-C4, 100% methanol was used as elution solvent in order to increase the protocol efficiency with tC_{18} cartridges with the stronger elution volume improving the percentage of drug eluted significantly in all drugs apart from MK-C3 (Figure 2.7).

For hydrophilic drugs (log P < 2), significant difficulties in effective drug recovery were encountered only in the case of metformin. Metformin is an extremely polar molecule which lacks hydrophobic functional groups. It was suggested that, due to the molecule's polarity, retention on the cartridge's hydrophobic functional groups was poor. While SDS conditioning did improve the recovery of metformin, the amount of drug recovered was still low (< 10%, data not shown). The most effective strategy in the case of metformin was the omission of the washing step. The omission of the washing step (tC_{18} and HLB cartridges), which improved the % recovery significantly (≈ 34% and 20% respectively-data not shown), was a far more effective strategy. Its combination with a change to a more hydrophilic cartridge like $C_{8,}$ a recovery value of 49.6 ± 1.9% was achieved (Figure 2.7). The functionalization of its silanol groups comprises of chains of eight carbon molecules instead of the eighteen like in tC_{18}, therefore it is suggested that the drug is retained to the column via weaker hydrophobic interactions with the stationary phase's silanol groups which are easier to break.

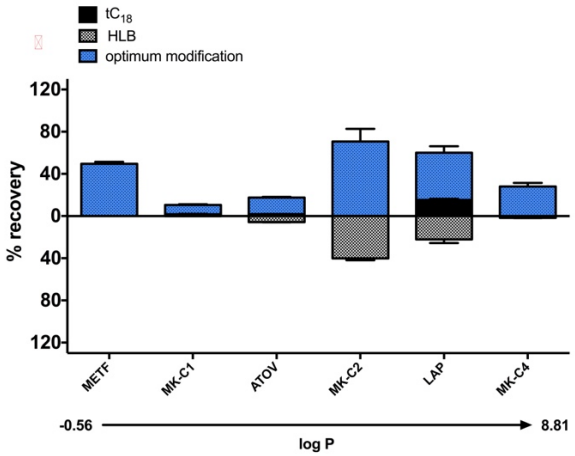


Figure 2.7. % recovery values of model drugs after modification of the standard SPE protocols.

Our observations for metformin and itraconazole (extremely hydrophilic and extremely lipophilic compounds respectively) (Figures 2.6a, b) were in agreement with previous studies which suggested that C_{18} cartridges are often a poor choice for drugs of extreme or poor hydrophilicity. Metformin [88] and itraconazole [89] were recovered by < 20% and < 40% respectively when eluted from C_{18} cartridges using methanolic solutions (studies in aqueous solutions and human liver microsomal' fraction respectively). Recoveries using the two different cartridges were significantly different in the majority of cases, ($p < 0.05$, 16/20 drugs), in the range of model compounds studied (Figure 2.6a), meaning that selecting one over the other can have a significant impact on the amount of drug to be recovered using a specific protocol in a study.

2.3.2.3. Prediction of the effect of physicochemical properties on extraction protocol (SPE) selection

The variables and their interactions of the PLS models examined are summarized in Figure 2.8. The PLS models constructed for % recovery values when tC_{18} and HLB cartridges were used (standard protocol) were defined by 1 and 3 Principal Components respectively. The PLS model developed for HLB was a good fit to the experimental values ($R^2 = 0.87$) and showed good predictive power ($Q^2 = 0.83$), while the model developed for the SPE extraction using a tC_{18} cartridge can only account for a low percent of Y variability ($R^2 = 0.34$), and has poor predictive power ($Q^2 = 0.24$). In both cases, the parameter having the most prominent positive effect was the log P*log aqueous solubility interaction (Figure 2.8), which is attributed to retention of a higher amount of drug in the SPE cartridge during the initial loading step, and also a more effective elution using a polar MeOH or MeOH/H_2O solvent in the elution step. In

tC_{18}, drug lipophilicity alone, but also its interactions with basic and neutral compounds, affected drug extraction negatively. The same applied for compounds which act as ampholytes in an aqueous environment while acidic compounds were easier to extract (positive standardised coefficient for weakly acidic compounds, VIP > 1). The recovery dependence of the compounds' ionisation state can be attributed to the presence of ionic interactions between charged drugs with the residual silanol groups of the cartridge which are unable to break with unbuffered MeOH:H_2O elution solvents [90]. Similar conclusions were deducted from the PLS regression model for HLB, with the difference being the negative correlation between log P and extraction efficiency, which was only present for unionised drugs in the working pH (log P*union fr interaction, Figure 2.8). The positive effect of aqueous solubility and its interaction with log P can be attributed to the more effective elution of polar compounds, when eluted with MeOH.

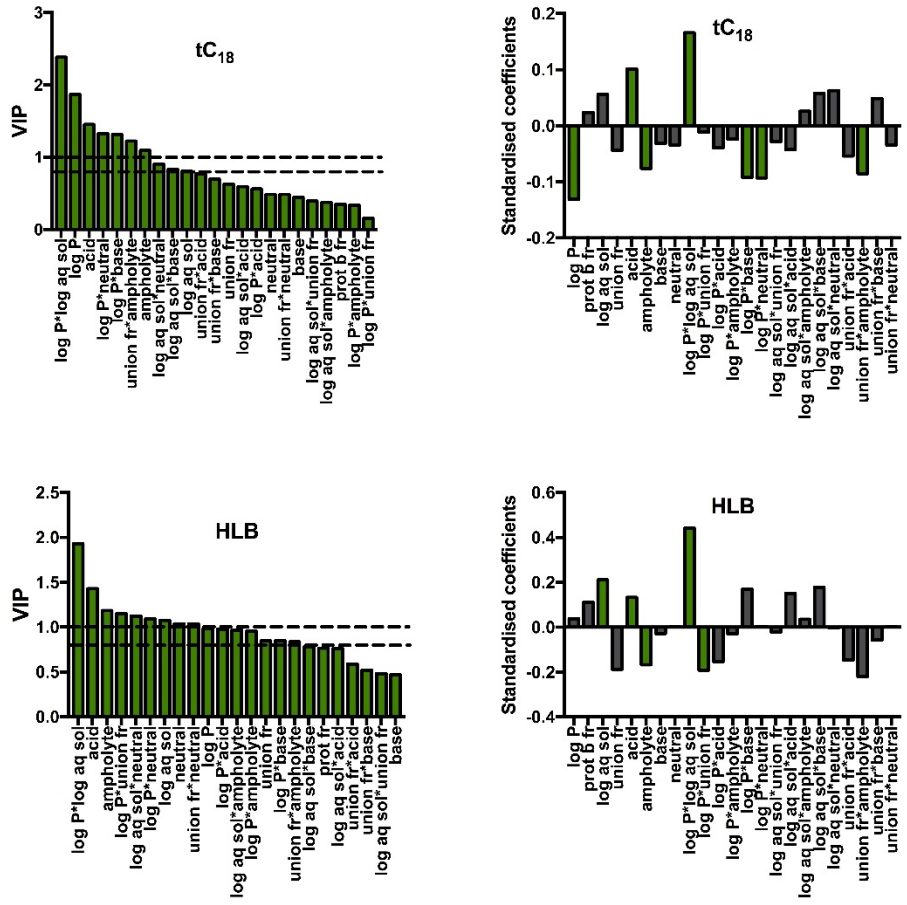


Figure 2.8. Variable importance in the projection (VIP) plot with the variables classed according to their importance of the response (left) for the selected SPE protocols. Standardised coefficients corresponding to the variables (and their interactions) studied. Green colour denotes coefficients of VIP values > 1, which are considered influential to the response value (right).

2.3.2.4. Designing a roadmap for effective sample treatment using solid phase extraction

In summary, tC_{18} and HLB cartridges can be effectively used for drugs of low to intermediate lipophilicity (log P = 0-5) while for extremely hydrophilic compounds, the use of C_8 cartridge and the omission of the washing step (where possible) were the most effective options (Figure 2.9). In the whole range of compounds, it was shown that both HLB and tC_{18} cartridges can be used, with HLB being more efficient for highly soluble drugs and also for weak acids, which are fully unionised at the working pH. Therefore, for highly soluble or weakly acidic compounds of log P between 0 and 5 the use of HLB cartridges is suggested. For drugs of extreme lipophilicity (log P > 5), increasing the strength of the elution solvent to 100% organic content usually increased the amount of drug recovered, but in certain cases recovery did not exceed 10-20% despite the attempted modifications of the initial protocol. Taking that into consideration, we suggest the use of 100% MeOH as elution solvent for compounds of extreme lipophilicity (using either one of the cartridges). HLB is suggested as the SPE cartridge of choice for the same reason as in compounds of moderate lipophilicity. For extremely lipophilic compounds (log P < 0), alternative cartridges could must possibly be used (e.g. C_8) for maximum efficiency, and if the medium permits, the washing step after sample loading in order could be omitted so as to maximise the amount of compound still retained on the cartridge before the elution step.

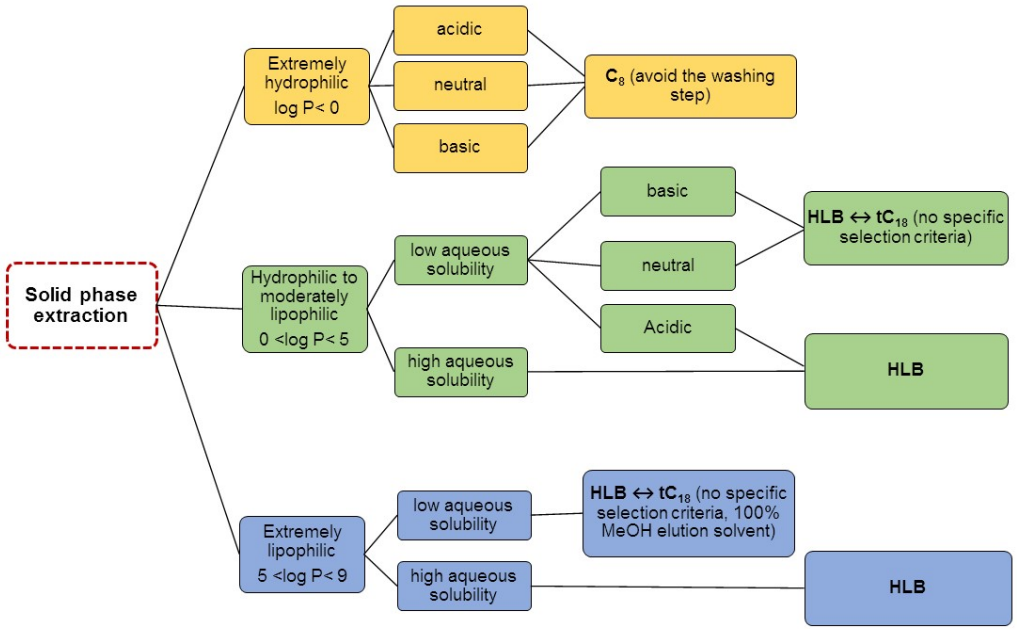


Figure 2.9. Roadmap of solid phase extraction conditions selected for maximum % drug recovery from the fed gastric medium.

2.4. Conclusion

Prediction of gastric food effect on drug absorption has been a big challenge for the pharmaceutical industry. Even though the *in vivo* properties of the fed state gastric environment have been quite well determined and some progress has been made with the development of gastric biorelevant media, a universal robust predictive analytical method has not been yet developed. The utility of such a method will allow the effective extraction and drug quantification of a range of drugs in heterogeneous fed biorelevant media selected on the basis of properties related to the medium, active ingredient or both. The above would drive drug analysis towards more standardised protocols and away from the current drug-by-drug assessment for optimal treatment conditions. The current study assessed the effective quantification of drugs, based on their physicochemical properties from milk-based media using two extraction techniques: i. Protein precipitation and ii. Solid phase extraction. The current study demonstrated that the use of three precipitation reagents (methanol, acetonitrile and 10% trichloroacetic acid) at a FeSSGF:reagent ratio of 1:2, when used according to the guidelines proposed, provided a simple sample preparation method which can be decided based on drugs' selected physicochemical properties. 10 % trichloroacetic acid was mostly suitable for weak bases of log P < 2, while either methanol or acetonitrile were effective for all the other model drugs. It has also been shown that the solid phase extraction protocols proposed using three different cartridges (tC_{18}, C_8 and HLB) provided good sample treatment methods for all drugs of a wide range of log P values (0.30–4) achieving recovery values > 69.5%. Modifications of the initial protocols, involving cartridge treatment and different elution solvents, improved the % recovery of the extremely lipophilic and extremely hydrophilic model drugs (9-60%), but with results still indicating that solid phase extraction is possibly not the method of choice for drugs of higher lipophilicity. Knowledge of the drug's key physicochemical properties is critical for the selection of the optimum extraction protocol for milk-based fed state media. In this study, the effect of the drug's physicochemical properties (lipophilicity, ionisation, aqueous solubility, protein affinity) and their interactions on recovery efficiency from fed state media were assessed, allowing the selection of the optimum extraction tool for drug quantitative analysis. The roadmaps developed for the two extraction techniques, can provide a starting point towards the development of a unified guideline, where selection of the extraction method can be made on the drug physicochemical profile. It is evident though that further studies are required for the elucidation of the analytical profile of a range of compounds in heterogeneous biorelevant media simulating the gastric fed state.

2.5. References

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Chapter 3: Impact of presence of excipients in drug analysis in fed-state gastric biorelevant media

Abstract

In this study, the impact of the presence of excipients in drug analysis in milk-based media which simulate the *in vivo* properties of the fed state stomach was investigated. 15 excipients, normally present in solid dosage forms of five APIs tested (atenolol, paracetamol, furosemide, nifedipine and propafenone hydrochloride) were mixed (one at a time) with the active pharmaceutical ingredient of interest either via vortexing, co-grinding or shaking of the physical mixture and dissolved in FeSSGF. The objective of the study was the assessment of the extraction efficiency of three protein precipitation protocols (using MeOH, ACN and 10% w/v TCA), typically used in drug analysis, in milk-based biorelevant media in the presence of the excipients. The efficiency of three different protein precipitation reagents in powder mixtures was compared against the equivalent drug amount recovered in the absence of the excipient in Fed State Simulated Gastric Fluid (FeSSGF). Most excipients had a significant negative effect ($p < 0.05$) on drug recovery in the milk-based medium as indicated by the multiple linear regression (MLR) analysis performed. For magnesium stearate and HPMC, the % recovery values were the lowest in four out of the five drugs studied, with a range of 10-100% depending on the API, mixing technique and protein precipitation protocol selected. The negative excipient-dependent effect was more profound in nifedipine and propafenone hydrochroride, the most lipophilic compounds of the study. Acetonitrile was the most effective protein precipitation reagent for most drugs in the presence of excipients, followed by methanol and 10% w/v trichloroacetic acid. Data analysis also revealed a dependence of the extraction method efficiency on the medium lipid content. Application of the above extraction protocols in commercially available formulations highlighted the need for assessment of the effect of excipients in extraction efficiency, before transferring the method directly to dissolution studies of formulations in milk-based fed gastric media. In conclusion, the presence of excipients and the selection of protein precipitation protocol are parameters which can affect significantly the efficiency of protein precipitation when FeSSGF is used as dissolution medium and need to be taken into consideration when developing a quantitative method based on the above sample clean-up technique.

Keywords: Fed state, Protein precipitation, Excipients, Formulation, Biorelevant media, Drug analysis

3.1. Introduction

Poor drug solubility has always been one of the biggest challenges the pharmaceutical industry has had to overcome. Approximately 40% of marketed drugs are classified as poorly soluble, while 90% of the drugs entering the screening process during drug development have a poor solubility according to Biopharmaceutics Classification System (BCS) [1]. Therefore, different formulation strategies were employed in order to improve the solubility and dissolution characteristics of the active pharmaceutical ingredients (APIs). Several cases where excipients such microcrystalline cellulose [2], kaolin [2], starch [3] and PEG 4000 [4] were used so as to improve the dissolution characteristics of poorly soluble drugs have been reported in the literature.

A second challenge concerns the media used for drug *in vitro* dissolution testing. Dissolution tests, as dictated by the United States Pharmacopoeia (USP) [5], cannot always provide information on the behaviour of the drugs *in vivo*, and therefore the need for multi-phase dissolution media able to simulate the gastrointestinal environment arose. The employment of media more "biorelevant", targets to simulate the passage of the formulation through the different compartments of the gastrointestinal tract both in the fasted and fed states. The use of such media can contribute towards the correlation of the results of the drug *in vitro* release with its *in vivo* pharmacokinetic performance (IVIVC), with an aim to decrease the number of *in vivo* studies conducted pre- and post-approval [6]. As far as gastric fed environment is concerned, the media developed could be classified in two categories: i. milk-based media, such as full-fat milk, digested milk and Fed State Simulated Gastric Fluid (FeSSGF) and ii. Lipid emulsions, such as Ensure®, Ensure Plus® and Intralipid® [7]. The media of the above two categories were developed as an attempt to simulate the gastric environment after the administration of a high or low fat standard breakfast respectively [8]. The composition of such media is constantly updated [9] and takes into consideration the protein/carbohydrate/lipid ratio and content as well as the fed gastric physicochemical properties measured *in vivo* [8, 10], making reasonable compromises. A disadvantage in the use of such media concerns their treatment prior to the sample analysis, which usually requires laborious steps for the extraction of drug before its quantification.

When a formulation is dissolved in such media, interactions may be formed between the excipients (excipient-excipient interactions) and also between the excipient and the active ingredient (excipient-drug interactions) or the dissolution medium (excipient-medium

interactions). Not only can such interactions affect the solubility and dissolution rate of the drug in biorelevant media, but they can also play a role in the design of sample clean-up techniques, the effectiveness of which may be compromised if only designed based on the physicochemical properties of the APIs. Therefore, an appropriate design would elucidate the role of excipients in the analysis in heterogeneous biorelevant media, such as the milk-based media used for the simulation of the gastric fed state in dissolution studies, with the current study focusing on the last two types of interactions. The type of interactions between drugs and excipients are described as physical or chemical [11], depending on their ability to induce chemical changes in the drug or excipient. Binding of drugs which have primary amine functional groups in the molecule to microcrystalline cellulose is a typical example of a physical interaction, leading to drug entrapment in the cellulose [12]. Primary amines can also interact with double bonds of certain excipients, like sorbitan monooleate via a reaction analogous to Michael addition, which is considered a chemical interaction. Changes in the excipient behavior, as a result of their interaction with the heterogeneous gastric environment, have been characterised a challenge which needs to be addressed in drug dissolution and analysis [13]. A known example of interactions between excipient and medium involves polyvinylpyrrolidone, which can form hydrogen bonds with water molecules through its carbonyl group [14], and undergo phase separation in aqueous 1.5 M KF solutions. In cases where the milk-based or lipid emulsion-gastric biorelevant media of interest are used in drug dissolution studies, such medium-excipient interactions may be even more complicated. The formation of protein and fat gel layers around hydroxypropyl methylcellulose (HPMC) matrices, which could potentially affect drug extraction when nutrient drinks or milk are used as dissolution media, is a typical example of medium-excipient interaction [15, 16].

In the present study, we investigated the impact of the presence of excipients in drug extraction when dissolved in the milk-based gastric fed state biorelevant media. Three hydrophilic (paracetamol, atenolol, furosemide) and two moderately lipophilic (propafenone hydrochloride and nifedipine) drugs were selected as model compounds. The excipients selected for the study consist of binders, lubricants, extended release matrix agents, emulsifiers, wetting agents and disintegrants. Hydroxypropyl methylcellulose is the most common cellulose used in hydrophilic matrices. It is used as a binder and also provides extended release characteristics to oral dosage forms [17, 18]. Magnesium stearate and stearic acid are used as tablet and capsule lubricants, avicel (microcrystalline cellulose) as binder and lubricant and povidone as binder, diluent and coating agent [18]. Tween 80 and sodium dodecyl sulfate are

employed as emulsifying, solubilizing and wetting agents [18]. Polyethylene glycols have various uses, such as suspending agents, co-solvents, binders, plasticizers or lubricants, depending on their solid state and molecular weight [18]. Croscarmellose sodium is used as disintegrant in tablets and capsules and Eudragit L100 and Eudragit E are brand names for polymethacrylate copolymers used as drug coatings for enteric drug delivery and taste masking respectively [19]. Finally, carbomer 974P is used as a binder and also as suspending, gelling and emulsifying agent [18]. Drug analysis in commercially available formulations was also assessed. The impact of excipients in drug analysis in a fed gastric medium was analysed using a regression analysis method [multiple linear regression (MLR)]. The study is a follow up of a previous study [20], where the impact of the active ingredients' physicochemical properties (log P, ionisation, aqueous solubility and protein binding) on analysis in FeSSGF using different extraction protocols (protein precipitation and solid phase extraction) was assessed. The objective of this work is the investigation of the impact of excipients in the efficiency (given by the percentage of drug recovered) of the protein precipitation protocols, developed and optimized for the analysis of the APIs. Except for the drugs' physicochemical properties, the current work aims to assess the dependence of drug-excipient mixing technique, protein precipitation extraction method, and dissolution medium's lipid content in an attempt to provide further insight towards the optimization of drug analysis in fed state media, and the analytical methods' application in formulations.

3.2. Materials and Methods

3.2.1. Materials

Furosemide (≥ 98% (HPLC)) and propafenone HCl (≥ 98% (HPLC)), were purchased from Sigma- Aldrich, UK. Nifedipine (98.0 to 102.0% (on dried substance)), paracetamol (97.5% min. (HPLC)) and atenolol (≥ 98% (TLC)) were purchased from Fisher Scientific, UK.

Sodium docecyl sulfate (≥ 99.0%) (GC)), povidone K30 (meets USP testing specifications), PEG 400 (202398), hydroxypropyl methylcellulose (H7509), were purchased from Sigma- Aldrich, UK. PEG 300 (Eur Pharm), stearic acid ((≥ 99.0%) (GC)) and PEG 6000 were purchased from Merck Millipore, UK. Microcrystalline cellulose (Ph-302) and croscarmellose sodium, (NF, Ph. Eur., JP) were purchased from FMC Biopolymers, UK. Carbomer 974P (Carbopol), PEG 4000 and magnesium stearate (Ph.Eur., BP, USP) were purchased from Fischer Scientific, UK. Tween 80 was purchased from VWR. Eudragit E (powder) and Eudragit L100 were purchased from Evonik Industries, UK.

Adalat® LA 30 mg tb and Arythmol® 300 mg tb were bought from Bayer, UK and Abbot Healthcare, UK respectively.

Cronus 13 mm regenerated cellulose (RC) syringe filters 0.45 μm were purchased from LabHut Ltd, UK. Whatman 13 mm glass microfiber syringe filters 2.7 μm (GF/D) were purchased from Fisher Scientific, UK.

Sodium acetate trihydrate, hydrochloric acid (36.5-38%), glacial acetic acid (≥ 99%), trichloroacetic acid 10% w/v and all phosphate salts were purchased from Fisher Scientific, UK. HPLC grade methanol, acetonitrile, trifluoroacetic acid (≥ 99.0%) and formic acid were all purchased from Sigma- Aldrich, UK.

0, 3.6 and 5% fat UHT milk was commercially purchased (Sainsbury's, UK).

3.2.2. Instrumentation

All samples were analysed in an HPLC system consisting of an Agilent 1200 series binary pump (G1312A), an Agilent 1200 series DAD detector (G1315D), an Agilent 1200 series autosampler (G1329A), an Agilent 1200 series controller (G1316A) with a Chemstation software (Agilent Technologies, Santa Clara, United States).

A pH meter Mettler-Toledo AG (model SevenCompact pH/Ion S220, Schwerzenbach, Switzerland), a centrifuge Hereus Biofuge Primo R (Thermo Scientific, Hanau, Germany) and a vortex mixer Rotamixer (HTZ, Cheshire, UK) were used.

3.2.3. Medium selection and preparation

Fed State Simulated Gastric fluid (FeSSGF) was selected as the working fed state medium due to its simplicity in its preparation. Its buffer capacity, osmolality and surface tension values are overall closer to the values measured *in vivo* after the administration of a standard meal than the equivalent properties of milk, which has been extensively used as a gastric fed state medium in dissolution studies [21]. FeSSGF was prepared according to Jantratid et al. [9] by mixing 3.6% fat milk and acetate buffer pH = 5 at a 1:1 volume ratio. Volume was adjusted to pH with 1 N HCl. Except for the standard version, two other different versions of FeSSGF were prepared, using 0% w/v ($FeSSGF_{sk}$) and 5% w/v ($FeSSGF_{hf}$) fat milk.

3.2.4. Selection of drug-excipient combinations

Assessment of the impact of excipients on % recovery in FeSSGF was conducted for five drugs (atenolol, paracetamol, furosemide, nifedipine, propafenone hydrochloride) selected from the study which involved the optimisation of extraction protocols for 20 APIs of a wide range of lipophilicity, ionisation and aqueous solubility [20]. Drug working concentration was defined as the drug dose dissolved in 500 mL of medium, unless limited by the solubility of drug in FeSSGF (Table 3.1). The current study assessed the effect of 15 excipients commonly used in commercial formulations of the above drugs (Table 3.2), using the optimised protein precipitation protocols for the quantification of active pharmaceutical ingredients developed in the previous study [20]. In summary, the extraction protocol involves addition of 2 parts of either MeOH, or ACN or 10% w/v TCA in 1 part of medium, brief vortexing (30 sec), centrifugation (8000 rpm, 15 min, 4 °C) and filtration through a regenerated cellulose 0.45 μm filter. The sample was diluted with "blank" acetate buffer pH 5 or MeOH:acetate buffer 1:1 pH 5, according to the drug solubility, where peak shape needed to be improved. The excipients used were selected based on the ones present in their commercial formulations as given in the Electronic Medicines Compendium (eMC) [22]. The percentage of the drug in the mixture was arbitrarily set as 30% w/w of the formulation. Extraction efficiency was given by drug absolute % recovery, expressed as per Eq. 2.1 below,

$$\%\ \text{absolute recovery} = \frac{\text{amount of drug in drug–excipient mixture FeSSGF solution}}{\text{amount of drug in standard FeSSGF solution}} \qquad \text{(Eq. 2.1)}$$

where the amount of drug in the presence and absence of excipient was quantified against calibration standards of the drug in FeSSGF, with both the mixture and drug standards treated with the same protein precipitation reagent.

Table 3.1. Physicochemical properties and working concentrations of model compounds.

Drug	log P [23-27]	pKa [28-32]	Working concentration (μg/ mL)
Atenolol	0.23	9.60	200
Paracetamol	0.46	9.50	200
Furosemide	0.74	3.90	80
Nifedipine	2.91	3.93	60
Propafenone hydrochloride	3.39	9.27	600

Table 3.2. Mixtures and ratios of excipients and APIs used. "✓" denotes the presence of each excipient and API in the mixture.

	Paracetamol	Furosemide	Propafenone hydrochloride	Nifedipine	Atenolol	Drug:excipient ratio in the mixture
Povidone K30	✓			✓		10
HPMC	✓		✓	✓	✓	0.6
Microcrystalline cellulose (Avicel)	✓	✓	✓	✓		1.2
SLS	✓				✓	20
Carbopol 974P				✓		6
Eudragit E				✓		3
Eudragit L100				✓		3
Magnesium Stearate	✓	✓	✓	✓	✓	10
Stearic acid	✓					10
Tween 80				✓		10
PEG 300					✓	10
PEG 400			✓			
PEG 4000	✓			✓		
PEG 6000			✓	✓		
Croscarmellose sodium		✓	✓			10

[a] Drug: excipient ratio value was selected within the range of % excipient concentration as dictated in "Handbook of Pharmaceutical excipients" [18].

3.2.5. Assessment of drug-excipient mixing process, type of medium and formulation analysis

The effect of the drug mixing process was assessed in a pilot study using three (atenolol, paracetamol and propafenone) of the five drugs. The mixing method of choice was selected on the basis of method robustness and lower data variability. An appropriate quantity of drug powder (10-200 mg) was mixed for 3 minutes with one related excipient at a time by either i. Using mortar and pestle, ii. Manual shaking in an Eppendorf tube or iii. Vortexing in an Eppendorf tube. API:excipient ratios were selected based on the percentage of each excipient in commercial formulations (Table 3.2), within the range dictated in the "Handbook of Pharmaceutical Excipients" [18]. PEG 300 and PEG 400, which are liquid, were mixed with the drug by manual shaking in a volumetric flask for 3 min before the addition of FeSSGF. A quantity of drug-excipient mixture, containing an amount of drug equivalent to the working drug concentration (Table 3.1) was transferred in a 200 mL volumetric flask and filled with FeSSGF. The flasks were incubated at 37 °C in a shaking water bath (200 shakes per min) for 90 min. Three 1 mL samples were taken from the top of each volumetric flask, filtered through GF/D filter, and each was treated with a different protein precipitation reagent (methanol, acetonitrile, 10% w/v TCA) as explained in the study for the extraction of the active pharmaceutical ingredients previously conducted [20]. The samples were filtered through 0.45 μm filters and were analysed using HPLC.

The same process was performed in the high fat ($FeSSGF_{hf}$) and no fat ($FeSSGF_{sk}$) versions of FeSSGF, in the drug-excipient mixtures where drug recovery was < 50% (where drug was extracted with MeOH) in order to assess the effect of the medium lipid content in drug recovery.

Commercial nifedipine and propefenone hydrochloride formulations (Adalat® LA tb 30 mg and Arythmol® tb 300 mg) were each placed in glass bottles filled with 500 mL of FeSSGF and were incubated at 37 °C under strong agitation (200 rpm) for 48 h so as to achieve maximum drug release. A sample was taken from each flask, filtered through a GF/D syringe filter and drug was extracted with the same precipitation reagent used for the physical mixtures and analysed as explained above. All experiments were performed in triplicate and % recovery was expressed as mean ± standard deviation.

3.2.6. HPLC analysis

Stock solutions of propafenone hydrochloride, nifedipine, atenolol, furosemide and paracetamol were prepared in MeOH. Calibration standard solutions were prepared in FeSSGF, FeSSGFhf and FeSSGFsk. The drugs were analysed in HPLC with the analytical methods (modification of published methods) used specified in the table below (Table 3.3).

Adsorption studies were performed in triplicate for each model drug for all types of filters used. No adsorption issues were observed for the drugs studied.

Table 3.3. HPLC methods (modification of published methods) used for the quantification of the model compounds.

Drug	Column	Mobile phase	Flow rate (mL/ min)	Temp (°C)	Inj. Volume (µL)	λ_{max} (nm)
Nifedipine [33]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 60:40	1	20	50	238
Furosemide [34]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Formic acid 0.1% v/v 60:40	0.8	25	20	233
Paracetamol [35]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 20: 80	1	10	20	257
Atenolol [36]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH: Phosphate buffer 0.01 M (pH = 4.5) 20:80	1	25	50	240
Propafenone HCl [37]	Agilent Eclipse XDB C_{18}, 120Å, 250 x 4.6 mm, 5 µm	MeOH:ACN: TEA:H_2O 50:7.5:0.1: q.s 100 pH = 2.9	0.8	25	20	248

3.2.7. Statistical Analysis

% absolute recovery and correlation with excipient type, mixing method and protein precipitation reagent used in drug analysis were evaluated in the context of a multi-way Analysis of Variance (ANOVA) with a post-hoc Bonferroni test (Statgraphics® v. XVI,

StatPoint Technologies Inc, US). Comparisons where $p < 0.05$ suggested a statistically significant difference.

The % recovery data was analysed via multiple linear regression (MLR) so as to investigate the impact of the selected excipient using SPSS v.22.0 (SPSS®, Chicago, IL, USA). Interactions of selected excipients with drug lipophilicity (log P) were included in the model on the basis of the lowest % recovery observed for the specific excipients (HPMC and magnesium stearate) in the pilot study. The generated MLR models were evaluated in terms of their regression coefficient (R^2) and variance inflation factor (VIF) with high R^2 values referring to a good fit to the model and VIF values < 3 indicating absence of multicollinearity among the independent variables [38]. The standardized coefficients of the factors plotted indicate the relative positive/negative effect on their corresponding values. The importance of each factor was evaluated but its p value. Statistical significance was set at $p < 0.05$.

3.3. Results and Discussion

3.3.1. Effect of drug-excipient mixing technique on % drug recovery in milk-based media (pilot study)

The contour graphs for the three model drugs of the pilot study express the % drug recovered as a function of type of excipient and mixing process (Figures 3.1a-c) for every protein precipitation reagent (MeOH, ACN, 10% w/v TCA) used for their extraction. It can be observed that % recovery was affected in all three drugs; an effect denoted by the colour change across the mixing process axis (y axis). For the three compounds selected, the mixing process had a significant effect on their % recovery after the medium's protein precipitation (Figure 3.2). Vortexing and grinding gave significantly different % recovery values ($p < 0.05$) in all three drugs (Figure 3.2), which implied that the powders' handling may affect their homogenous mixing.

As observed in Figure 3.1a, the % recovery of paracetamol was close to 100% in the vast majority of cases with all reagents, excipients and mixing methods (mean paracetamol recovery = 100.9 ± 9.6%), a fact possibly attributed to paracetamol's increased wettability. Contact angle measurement is a method of determination of the wettability of a compound, with angles > 90° being indicative of poor compound wettability [39]. The measured contact angles of paracetamol's polymorph commercially used, monoclinic form I [40], against water ranged from 15.9° (± 3.1°) to 67.7° (± 2.5°) depending on the crystal facet [41].

The % recovery values of furosemide (Figure 3.1b) ranged between 40.9 and 110.2% depending on the excipient and extraction reagent used for drug recovery. Water's contact angle against furosemide has been measured > 90° and could provide a reason for the lower recovery values compared to paracetamol [42].

In both hydrophilic drugs (paracetamol and furosemide), grinding process led to lower recovery values than in the other two mixing processes (Figure 3.2). Grinding is a common strategy used to reduce drug particle size, often applied to drugs of poor solubility with an aim to increase their bioavailability *in vivo* [43], and although co-grinding of drugs with several excipients, such as lactose monohydrate [44] and avicel [45] accelerated their dissolution profile, there are cases when co-grinding may be used to prolong dissolution and lead to a sustained release profile [46]. A study with theophylline, a hydrophilic compound with log P value similar (log P = -0.02 [47]) to the above drugs, demonstrated that co-grinding with magnesium stearate decreased the dissolution efficiency and mean dissolution rate of the formulation [48] in comparison to a physical mixture of the same powder quantities. Therefore, a reason for the decreased drug % recovery when an excipient more hydrophobic than the active ingredient and the drug are co-ground could possibly be the concentration of hydrophobic particles of decreased particle size around the API, leading to delayed drug dissolution in the fed state medium.

The extraction values of propafenone hydrochloride, a lipophilic drug, were lower than in the two hydrophilic drugs described above (Figures 3.1c, 3.2), and had a wider range. % recovery values were as low as 17 ± 7.9% (HPMC/shaking mixing method/drug extracted with 10% w/v TCA). In this case, grinding led to significantly higher drug recovery values than in the other two mixing methods, despite the fact that some of the excipients were common in the other drugs as well (HPMC, magnesium stearate), which suggests that the extraction efficiency in the milk-based medium depends both on the API and ingredient's properties.

The decrease in particle size and the possible alteration of the powders' surface properties when ground with mortar and pestle were two parameters which had to be taken into consideration in assessing the effect of excipients and protein precipitation solvent. Therefore, vortexing was selected as the powder mixing method for the assessment of the above parameters in all drugs and excipients. Despite showing equally high deviation between samples with the others it was more easily controlled than manual shaking (constant stirring

speed) and provided homogenous mixing with minimal changes of the powders' surface properties, unlike grinding.

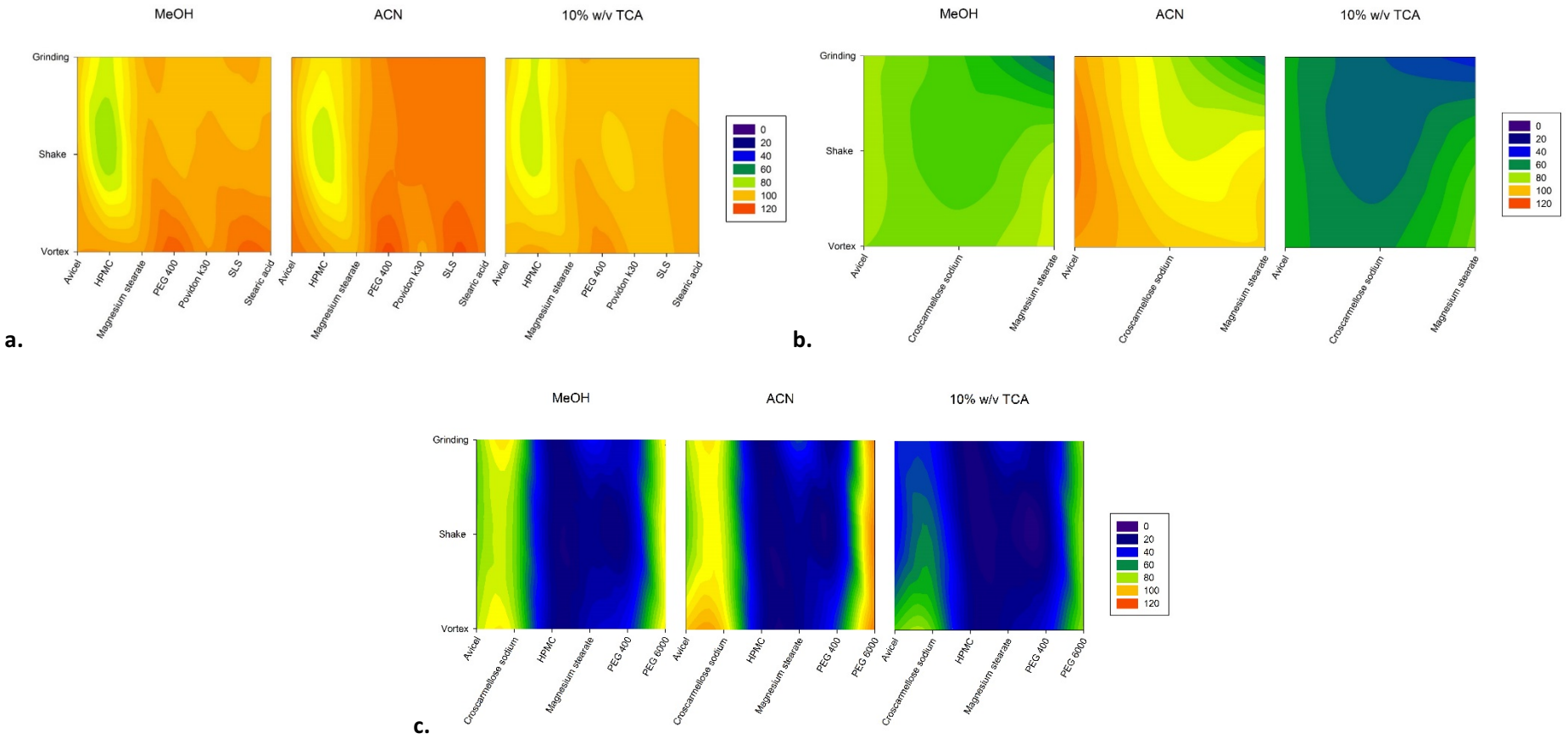

Figure 3.1. Drug-excipient gradient maps for **a.** paracetamol, **b.** furosemide and **c.** propafenone hydrochloride. Contour plot of % recovery values as a function of drug-excipient mixing method and excipient. "Warm" colour regions (yellow, red) indicate that amounts of drug similar to their theoretical recovery values in the absence of excipient (% recovery > 80%) were able to be extracted from the medium, whereas "cold" regions (blue, purple) are indicative of poor drug % recovery.

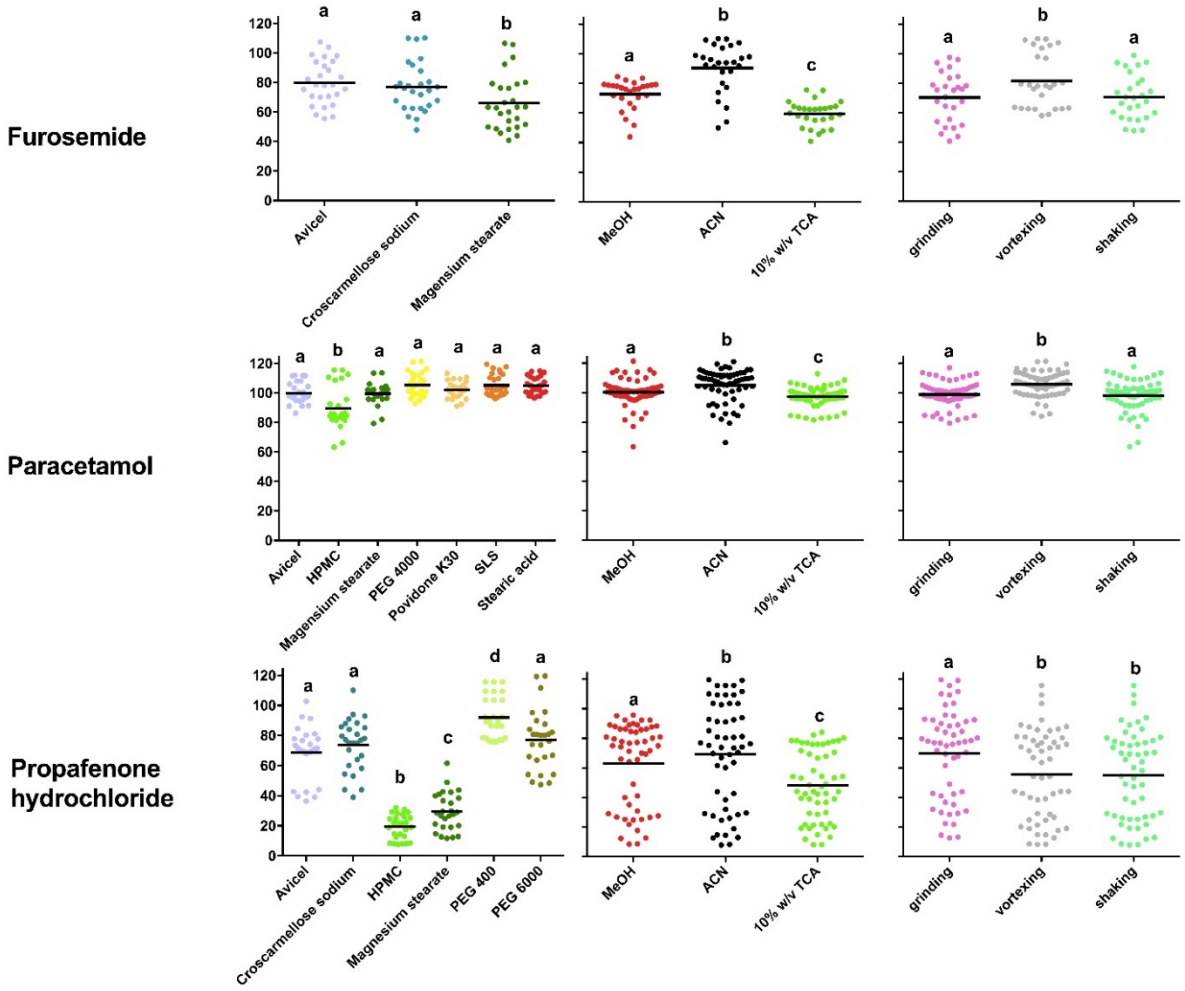

Figure 3.2. Three-way ANOVA of excipient, protein precipitation reagent and mixing method effects (from left to right) on drug % recovery for the three model drugs. Different letters denote a statistical difference in the % recovery between excipients, reagents or mixing processes ($p < 0.05$).

3.3.2. Effect of excipient type and extraction reagents (protein precipitation solvents) on drug % recovery in milk-based media

The protein precipitation results of the full study (mixtures of the 5 drugs with the 15 excipients) are presented in Figure 3.3, with the drug % recovery expressed as a function of combined excipient and drug. Drugs are sorted by increasing log P values from bottom to top. Multiple linear regression analysis of the results of the full study, in combination with the multiple comparisons of the pilot study were used to evaluate the effect of the excipients and protein precipitation solvent on % drug recovery. Overall, the results of the MLR analysis of the drug-mixtures excipients for the extraction of each protein precipitation solvent showed good fits with high R^2 values (0.76, 0.66 and 0.7 for the extraction with MeOH, ACN and 10% w/v TCA respectively) and VIF values < 3 for the independent variables of the model (Figure

3.4). The analysis demonstrated statistical significance for most of the variables tested ($p < 0.05$).

3.3.2.1. Excipient effect in drug recovery from milk-based media

The pilot study performed with the three drugs, revealed that the % recovery is both API and excipient dependent. The multiple comparisons' test performed with the three drugs showed that in all cases, their mixtures with magnesium stearate and HPMC led to significantly different recoveries compared to the rest of the drug-excipient mixtures, with lower mean % recovery values (Figure 3.2). When the excipient effect was evaluated for all five drugs (vortexing), decreased % recovery values were observed in more drug-excipient mixtures in addition to the two mentioned above, denoted by the blue and green zones in the contour plots of the vortexed mixtures (Figure 3.3). Furthermore, most excipients showed a statistically significant negative effect on % drug recovery, as demonstrated by the results of the MLR analysis (Figure 3.4), with the excipient effect discussed for each drug separately below. From the contour map of the full study (Figure 3.3), it can be observed that the recovery values for the three hydrophilic drugs (atenolol, paracetamol, furosemide) were distinctively higher than in the mixtures of the lipophilic ones (nifedipine, propafenone hydrochloride).

In the case of paracetamol, none of the excipients decreased % drug recovery more than 15% except for HPMC, which was attributed to the drug increased wettability as reported above. When HPMC was mixed with other active ingredients as well, (atenolol, nifedipine, propafenone hydrochloride) the % recovery of the drugs ranged from approximately 80 % for paracetamol down to approximately 20% for propafenone hydrochloride, regardless of the extraction solvent of choice (Figure 3.3). In the case of atenolol, similar results were observed. The effect of PEG 300 and SLS in drug recovery was minimal (> 79.5% in all mixtures). In the cases of HPMC and magnesium stearate, recovery values as low as 61.3 and 56.9% were observed for the two excipients respectively. In furosemide mixtures, the drug recovery was mainly controlled by the extraction reagent, rather than the excipient, an effect discussed in the section below. Specifically, the % recovery in the presence of avicel, croscarmellose sodium and magnesium stearate were all approximately 60, 80 and 100% when 10% w/v TCA, MeOH and ACN were used for the extraction of drug from FeSSGF respectively (Figure 3.3).

One possible reason for the lower recovery in the presence of HPMC could be the formation of a barrier of fat and/or proteins formed around the powder in the milk-based medium, decreasing medium permeation [49] to the inner part of the formulation, as reported

in several studies where nutrient drinks like Ensure Plus® or Nutrison® were used as fed gastric dissolution media [15] [50]. It was also demonstrated that the initial gel formation layer of HPMC matrices during dissolution increased according to the fat percentage of the fed state medium, although the difference could be attributed to other properties of the medium, such as its viscosity [16]. Magnesium stearate acts by preventing the adhesion of the powder during tablet compression, forming a non-uniform hydrophobic layer on the surface of the powder mixture [51, 52]. Therefore, the decreased % recovery values in all drugs mixed with magnesium stearate could be attributed to the slower drug dissolution in the medium, due to the excipient's hydrophobic nature [53]. Interestingly, although most excipients demonstrated a significant negative effect on drug % recovery (green bars), and HPMC and magnesium stearate showed a negative effect in the multiple comparisons test of the pilot study, they did not have a statistically significant contribution to the MLR final model (Figure 3.4). Their interactions with drug lipophilicity though demonstrated a highly significant effect, as shown by the high standardized coefficients of the respective variables (HPMC*log P, magnesium stearate*log P) (Figure 3.4). Therefore, the negative impact of HPMC and magnesium stearate on drug recovery is more profound in drugs of high lipophilicity (log P) and can attributed to the possible formation of layers around the drug powder either self-induced or in combination with the milk-based medium [15, 51, 52].

As far as the effect of the excipient on the dissolution of the lipophilic drugs is concerned, the short duration of the study (90 min) seems to be the most probable reason for the log P/excipient-dependent % recovery. Most of the excipients appear to have a log P-dependent effect on the dissolution of the APIs, meaning that a time > 90 min would be required for the total amount of lipophilic drugs to be solubilized. The combinations of nifedipine with all excipients demonstrated % recovery values < 80%. Except for the HPMC and magnesium stearate, the effect of which on drug dissolution was previously described, the lowest values reported for nifedipine were in its mixtures with Eudragit L100, Eudragit E and carbopol 974P. Eudragit L100 is insoluble below pH 6, according to the manufacturer, which could result in co-precipitation of the drug in the FeSSGF of pH= 5. Eudragit E is soluble at gastric fluid of pH up to 5, meaning that in the working pH, after the addition of methanol or acetonitrile, precipitation of the excipient along with the drug could take place due to the pH of the supernatant (pH of the supernatant measured > 5). The reason of the decreased drug recovery values when APIs were mixed with carbopol 974P can be attributed to the same reason as HPMC and magnesium stearate; the formation of a gel layer around the particles of the active

ingredient decreasing the diffusion coefficient of the drug in the medium [54]. Another possible mechanism suggesting reduced drug transport to the dissolution medium due to interaction between the drug and the polymer has also been reported in the literature [55]. Avicel's negative effect on nifedipine recovery (< 60% with all protein precipitation reagents) (Figure 3.3) could also be attributed to the entrapment of the smaller drug particles between the microcrystalline cellulose's particles leading to slower drug wetting and dissolution [56]. Water soluble polyethylene glycols (PEG 4000, PEG 6000) and Tween 80 were normally expected not to affect % drug recovery negatively, as they act as solubility enhancers [57] and surfactant [58], improving the solubility and dissolution characteristics of poorly soluble drugs. Therefore, the low values observed for all excipients and the big differences in drug recovery among different protein precipitation solvents (maximum drug recovered = 31.7% with 10% w/v TCA) point in a recovery in this case being log P- or extraction solvent-driven, rather than excipient type-dependent. A similar explanation could be given for the negative effect of povidone K30, a hydrophilic polymer which has been shown to increase both wettability and dissolution rate of lovastatin, a drug of similar lipophilicity to the lipophilic drugs (nifedipine, propafenone hydrochloride) of the study [59] (log P = 4.26 [47]). Finally, similar results were observed in the mixtures of propafenone with its excipients, with the drug % recovery in the presence of avicel, croscarmellose sodium and polyethylene glycols affected negatively, driven by the excipient- and log P-related mechanisms described above. In the case of this more lipophilic drug, drug recovery in the presence of HPMC and magnesium stearate was significantly lower than in the presence of the other excipients ($p < 0.05$) (Figure 3.2). % recovery values were the lowest in the presence of the former, and did not exceed 40% regardless of the choice of the extraction solvent. The challenging nature of the extraction of lipophilic drugs from HPMC matrixes has been reported and attributed to the high API lipophilicity and the gelation properties of the polymer [60], an effect demonstrated in the current study too, as indicated by the high negative HPMC*log P standardised coefficients in MLR analysis (Figure 3.4).

3.3.2.2. Protein precipitation reagent effect in drug recovery from milk-based media

For the three hydrophilic drugs (atenolol, paracetamol, furosemide), recovery values > 60% were observed for all three reagents (Figure 3.3), with the highest recovery observed in ACN extraction, as the red zones of the graph (Figure 3.3) indicate. In regard to the lipophilic drugs (nifedipine, propafenone hydrochloride), the amounts of drug extracted were distinctively lower for all three reagents. Particularly for 10% w/v TCA, the percentage of drug

recovered in the presence of excipients was extremely low; less than 40% of the drug was recovered in 15 of the total 16 nifedipine/propafenone hydrochloride and excipient mixtures (Figure 3.3). At the TCA concentrations used, unfolding of the medium's proteins is set off by negatively charged ions of the acid, which cause the disruption of the electrostatic forces maintaining the structure of its proteins. This mechanism of action can potentially expose non-polar protein surfaces and lead to plotting of the molecules and precipitation [61]. It is possible that the presence of excipients may inhibit the solubilisation of the drugs by the acid and facilitate its occlusion in the precipitate.

The effect of the type of protein precipitation solvent was statistically significant in terms of % recovery ($p < 0.05$), as demonstrated by the ANOVA analysis of the pilot study (Figure 3.2). The differences can be observed in the different reagent levels of the visual ANOVA representation (Figure 3.2) and also in the different colour zones of the contour graphs (Figure 3.3); the reddest zones observed belong to ACN and the bluest to 10% w/v TCA, indicating the highest and lowest recovery values respectively. The above order of extraction efficiency (ACN > MeOH > 10% w/v TCA) was followed in four of the five drugs regardless of excipient of mixing method, except for atenolol as observed in the contour plots (Figures 3.1, 3.3). In the case of atenolol mixtures, which was the only drug more poorly extracted with ACN than with TCA, the better applicability of trichloroacetic acid cannot be directly justified by the drug's physicochemical properties, as in the absence of excipient both ACN and 10% w/v TCA were able to recover approximately 100% of the drug in FeSSGF [20]. It could be suggested that drug's comparatively higher solubility in trochloroacetic acid than in acetonitrile [62] led to faster drug solubilisation in its mixture with the excipient.

It is evident that the properties of the active ingredients alone cannot account for the differences in recovery among the different extraction methods, as the reference standard for 100% recovery was defined for each reagent separately and given by the amount of drug recovered from the medium in the absence of its excipient. It is worth mentioning, that the extraction efficiency (in terms of effective drug recovery) of the three reagents used in the presence of excipients, followed the same pattern as in their absence, as demonstrated in a previous study [20]. In both studies, drug lipophilicity had a negative effect on the amount of drug recovered, which may suggest that the presence of excipients may amplify the differences in drug recovery, previously correlated with the drug's physicochemical properties. Therefore, the physicochemical properties of the API need to be taken into consideration in the design of an effective sample clean-up method, both in the absence and presence of excipients.

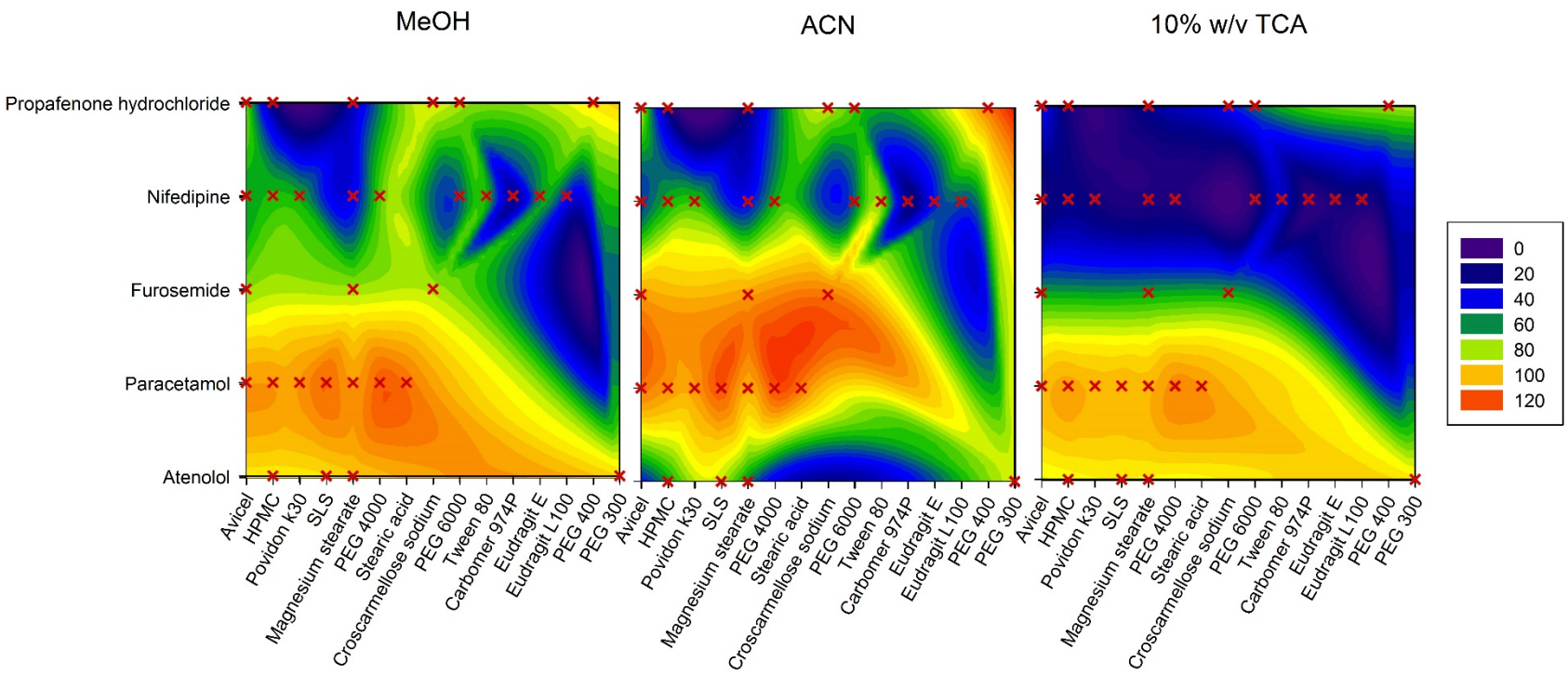

Figure 3.3. Drug-excipient gradient map (mixed by vortexing). Contour plot of % recovery values as a function of drug and excipient. Red x points denote the drug-excipient combinations used in the mixtures.

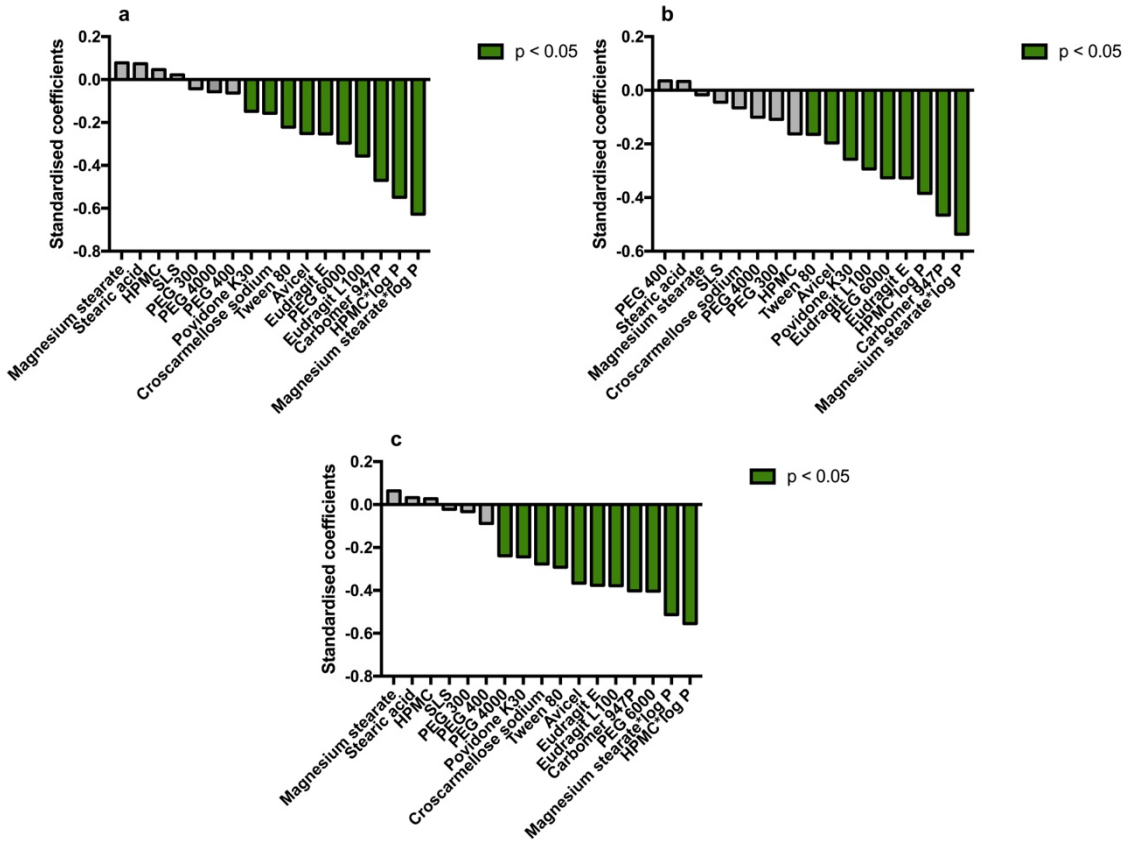

Figure 3.4. Standardised coefficients of MLR analysis after protein precipitation with **a.** MeOH, **b.** ACN and **c.** 10% w/v TCA. Green colour denotes statistical significance.

3.3. Effect of medium fat content on drug %recovery in milk-based media and assessment of drug extraction method in solid oral dosage forms

Analysis in media of different fat content was performed for the two lipophilic drugs of the study, nifedipine and propafenone hydrochloride, in mixtures with excipients demonstrating the low (< 50%) drug % recovery (magnesium stearate, carbomer 974P, Eudragit L100 and HPMC).

The recoveries in the high and no fat media for the excipient mixtures with nifedipine were equally low. Nifedipine recovered ranged from approximately 3 to 16% and from 4 to 18% for the low and high fat medium, while the equivalent values for FeSSGF gave values from 7 to 50% for the three reagents used (Figure 3.5). The reduced values in the low fat medium, compared to FeSSGF, were attributed to the lower drug solubility. Nifedipine's solubility in FeSSGF is approximately 70 μg/mL [63]; a value approximately 7 to 10-fold higher than the drug's aqueous solubility, with the difference possibly attributed to the solubilisation of the lipophilic drug in the lipid portion of the medium. Therefore, the absence of fat in the medium may be the reason for the lower drug solubility and consequently lower

recovery. In the high-fat medium, low recovery values are possibly attributed to the decreased extraction efficiency in media of high fat content, a common issue of sample clean-up methods in milk-based media [7]. A study of HPMC-nimodipine mixtures in acetate buffer pH = 4.5 (log P = 3.41 [64], similar structure and lipophilicity to nifedipine) showed an increase in the drug solubility and dissolution efficiency by a factor of 4 compared to the drug in the absence of the excipient [65]. This which points that low solubility and dissolution rates cannot always be attributed to the excipient but to the interactions between the mixture and the dissolution medium as well. In the case of propafenone, medium prepared with skimmed milk ($FeSSGF_{sk}$) gave higher drug recovery values when ACN and TCA were used as protein precipitation reagents (Figure 3.5), which was again attributed to the difficulty of designing an effective extraction protocol in media of high lipid content.

Extraction of drug of commercial formulations revealed that despite the presence of the excipients, their extraction was in no case affected to such an extent as with the simple mixing of each excipient separately. The % recovery value was in all cases between 67.2 and 99.5% (Figures 3.5a, b) and its dependence of the solvent selection profound, in the same way as with the physical mixtures of APIs and excipients. Acetonitrile was most effective, followed by methanol and 10% w/v TCA. The reagent-dependent recovery results in drug formulation indicate that suitability of the extraction method for the active ingredient does not necessarily guarantee equivalent extraction performance in formulation analysis. The reduced recovery in extraction with trichloroacetic acid may indicate entrapment or adsorption of the drug in excipients, and inability of the method to break these excipient-drug interactions. A use of such a method without prior assessment of drug-excipient interactions could lead to erroneous results if potentially used for drug quantification in a dissolution study using milk-based media. The differences in recovery values among formulations and reagents, along with the results of the assessment of the mixing technique, confirmed that the process via which drugs and excipients are formulated has to be considered for the development and optimisation of a sample clean-up protocol.

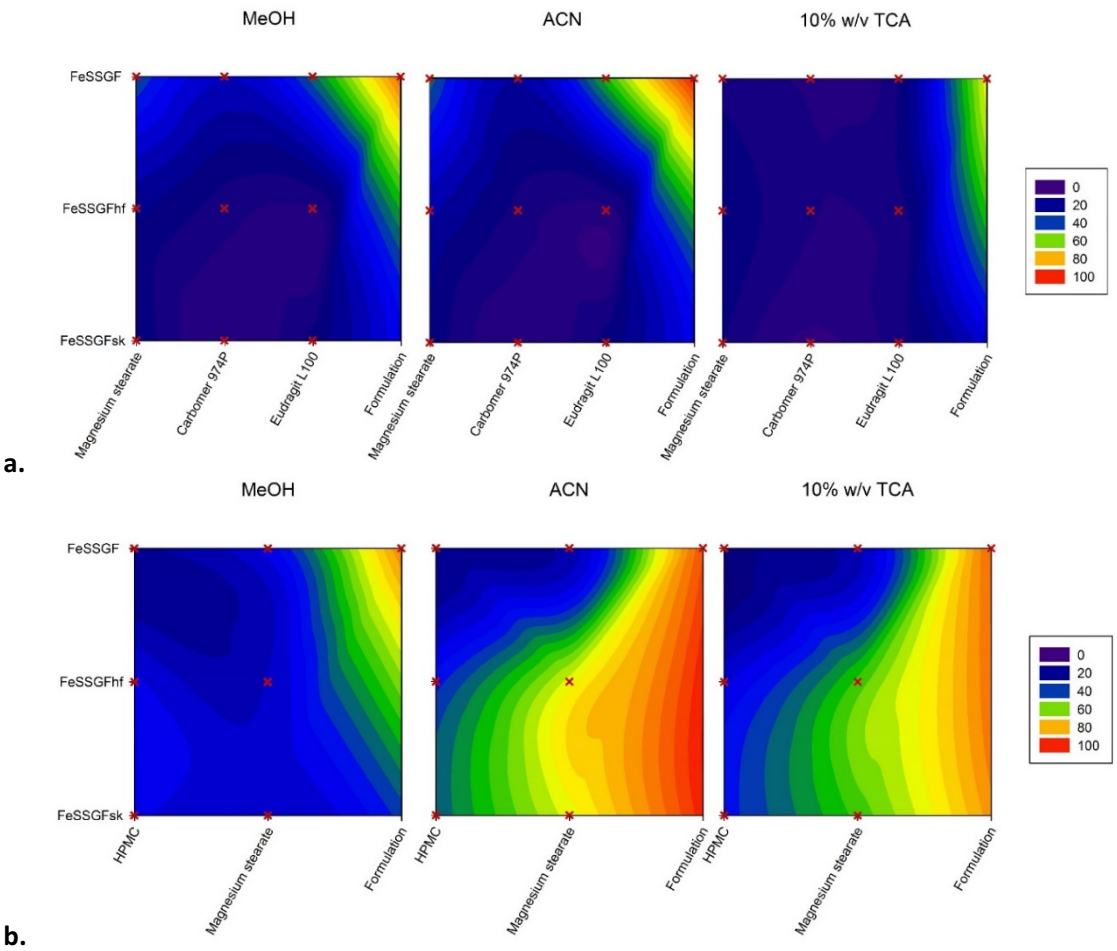

Figure 3.5 a. Nifedipine-excipient and **b**. propafenone-excipient mixtures, media and commercial formulations gradient map. Contour plot of % recovery values as a function of medium and excipient/formulation. Red x points denote the drug-excipient-medium combinations.

3.4. Conclusion

The presence of excipients can have an effect on the amount of drug extracted from milk-based gastric media and should therefore be taken into consideration when developing a quantitative method for drug analysis. Using a previously developed extraction protocol [20], the effect of excipients used in commercial formulations in the recovery of drugs when dissolved in milk-based fed gastric media was investigated. The results demonstrated dependence of the type of excipient, mixing technique and protein precipitation protocol selected with the interaction between lipophilicity and certain excipients (HPMC, magnesium stearate) being highly influential in most cases, as indicated by the MLR analysis. The differences in the impact of the same excipients in drugs of different lipophilicity highlighted the need for further investigation of excipient-drug interactions and the way both the excipients' and APIs' physicochemical properties can affect drug analysis in fed state gastric

media. The study revealed a medium-dependent recovery in the presence of excipients, but without indications of a direct correlation between medium's fat percentage and amount of drug recovered. Finally, it was concluded that excipient processing during drug manufacturing may affect the efficiency of the sample clean-up methods, and has to be taken into consideration in drug analysis and quantification. Therefore, to accomplish the accuracy required in drug analysis in fed state milk-based media, the effect of drug properties, type of excipient, changes in medium composition and formulation manufacturing have to be individually assessed in regard of their effect on the extraction efficiency. Further studies which will assess the full applicability of the optimized extraction protocols developed for the active pharmaceutical ingredients on the different types of drug formulations are required.

3.5. References

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Chapter 4: Investigation of drug partition kinetics to fat in simulated fed state gastric conditions based on drug properties

Abstract

The presence of fat in the gastric environment can affect the pharmacokinetic behaviour of drugs with mechanisms which have not been yet fully understood. The objective of the current study was to assess the drug partition to the lipid part of the fed gastric content under different emulsification conditions, using *in vitro* discriminating setups. The model drugs used in the study were selected on the basis of different physicochemical properties (lipophilicity, ionization, molecular weight and aqueous solubility) and different food effect observed in *in vivo* human studies. Fed State Simulated Gastric Fluid prepared with skimmed milk ($FeSSGF_{sk}$) and anhydrous milk fat were used as surrogates for the aqueous and fat portions of the fed gastric environment respectively. An optimized biphasic model was developed so as to predict the differences in partition rates to fat, for model drugs of a wide range of the properties mentioned above. Molecular weight, molecular weight and log $D_{pH\ 5}$ interaction and negative food effect act as negative factors to the rate of fat partition, while absence of food effect and $logD_{pH\ 5}$ interaction with aqueous solubility affect the rate of partition to fat favourably.

Keywords: Fed state, Physicochemical properties, Food effect, Drug partition, Partial least squares regression

4.1. Introduction

The oral route is considered the most common route of drug administration, due to its convenience, lower cost of formulations developed and patient compliance. Drug solubility, dissolution and permeability are critical processes taking place in the gastrointestinal tract and determine the drug's bioavailability. For most solid dosage forms (with the exception of orodispersing formulations), the gastric environment is the part of the GI tract where dissolution begins as the volumes and transit time in the oral cavity and oesophagus are insignificant. The stomach can be divided in three distinct parts: fundus, corpus and antrum; the fundus acts as a gastric reservoir, the antrum is the site where trituration and particle size reduction takes place, while the corpus connects these two parts [1]. Food forms layers in the stomach, with fat floating on top of an aqueous layer and heavier particles sedimenting in the sinus [2], while the aqueous layer contains small particles which are emptied from the stomach as the gastric emptying process takes place [1]. The lipid part of the meal administered has a prominent role in a potential drug food effect through many possible mechanisms. Some common ones involve the increase of lymphatic transport of drugs [3], the constriction of intestinal efflux transporters and the formation of intestinal mixed micelles (bile salt/phospholipids/cholesterol) as a result of exogenously administered lipids [4]. The mechanism affecting the food effect of drugs which is related to the presence of fat in the stomach, mainly involves the increase in gastric residence time of drugs which thus allows more time for drug dissolution. Therefore, the presence of lipid components in meals can modify the *in vivo* behaviour of the certain molecules either promoting the formation of mixed micelles with bile salts or by drug solubilisation by fat.

Thus, when developing *in vitro* predictive tests towards the evaluation of drug dissolution in the GI tract it is important to simulate the effect of the lipid part of the meal, incorporating it to the dissolution media used, with an aim to predict effectively possible food effects on their pharmacokinetic behaviour. Since fat can improve the dissolution characteristics of poorly soluble drugs in the stomach, the knowledge of possible drug-lipid interactions is essential. Also, knowing the rate that the drug partitions to fat is equally important, as the meal remains in the gastric compartment for 1-4 hours (depending on the type of meal) [5], with one part of the released drug being dispersed in the lipid phase and the rest solubilised or precipitated in the aqueous gastric phase. Of the total solubilised amount of drug, which includes both the free and partitioned drug, only the free fraction has the potential to be absorbed [6]. It is therefore important to determine the parameters which control drug-lipid

interactions and evaluate possible dependence of drug physicochemical properties. Knowledge of the physicochemical parameters controlling this type of interactions can shed light towards the understanding of the mechanisms inducing positive or negative food effect after drug co-administration with high-fat meals.

Another factor which can affect drug dissolution and permeability in the gastrointestinal tract is the presence of lypolytic enzymes. Lipid digestion starts in the oral cavity with the help of lingual lipases and continues in the stomach from lingual and gastric lipases; while the major part of the lipolysis process takes part in the small intestine, where drug absorption takes place, by the pancreatic lipase [7]. Gastric lipolysis was believed to account for 10-20% of the total lipolysis process in the GI tract [8, 9]. More recent evidence though, showed that human gastric lipase (HGL) may be responsible for up to 40 % of the total lipolytic activity [10]. Gastric lipase hydrolyses long and medium-chain to diglycerides, monoglycerides and fatty acids. These digestion products along with the shear forces developed in the stomach during digestion lead to fat emulsification, creating a coarse lipid emulsion [11]. Because of the limited role of HGL in the dissolution of conventional tablets, its use in gastric dissolution media has been relatively limited. Its presence though may be important for lipid-based drug delivery systems [12] and possibly in the prediction of drug dissolution behaviour after administration of lipid rich meals, as these ezymatic processes take place in both cases (lipids derived from food or lipid based formulations) [13]. In the fed state, gastric lipase contributes to a greater extent to the total lipolysis, due to higher HGL stimulation at higher pH values, with its activity measured more than 10 times higher at pH=5.4 than at pH=2.8. [12].

In the fed stomach, un-digested fat forms a lipid layer floating on the top of the gastric content while emulsified fat particles move with the aqueous phase to the pyloric antrum; the aqueous content fills the distant antrum and moves towards the duodenum faster than fat and solid residues [2]. Because of the complex stratification of aqueous phase and un-digested and emulsified fat in the fed stomach, it is important to assess the interactions formed between the drugs and each of the phases so as to explain certain lipid-induced changes in drugs' pharmacokinetic parameters and also problems in the analysis of biorelevant media related to the presence of fat.

The current study, aimed to determine not only the percentage of drug partitioned to fat from the "aqueous" phase of the stomach under physiological conditions in the fed state, but also the differences in the rates at which the above process takes place among different

drugs. The purpose of the current work is to study the role of the lipid part of a meal, simulated in a gastric fed state medium, in inducing changes of drug pharmacokinetic parameters. Finally, the study assessed the impact of related drug physicochemical properties and drug food effect observed *in vivo* (changes in the drug's pharmacokinetic behaviour after meal administration) on drug partition using statistical tools [partial least squares (PLS) regression analysis].

4.2. Materials and Methods

4.2.1. Materials

Atorvastatin calcium salt trihydrate (≥ 98% (HPLC)), danazol (≥ 98%), furosemide (≥ 98%), phenytoin (pharmaceutical secondary standard; traceable to USP and PhEur), itraconazole (≥ 98% (TLC)), propafenone hydrochloride (≥ 98% (HPLC)), indomethacin (≥ 99%) and indoprofen (analytical standard) were purchased from Sigma- Aldrich, UK, while nifedipine (98.0-102.0% (on dried substance)) and ketoconazole (inclusive between 98.0%) from Fisher Scientific, UK. Griseofulvin (> 97%) and felodipine were purchased from Alfa Aesar, UK and ibuprofen (97-103%) was purchased from Fagron, UK. MK-C1 and MK-C4 were provided by Merck & Co, US.

Sodium acetate trihydrate, sodium chloride, hydrochloric acid (36.5-38%), glacial acetic acid (≥ 99%), sodium dodecyl sulphate (S/5200/53) and all phosphate salts were purchased from Fisher Scientific, UK. HPLC grade methanol, acetonitrile, trifluoroacetic acid (≥ 99.0%), diethylamine (≥ 99.5%), thriethylamine (≥ 99.5%) and formic acid were all purchased from Sigma- Aldrich, UK.

Cronus 13 mm regenerated cellulose (RC) syringe filters 0.45 μm were purchased from LabHut Ltd, UK and 2.7 μm (GF/D) filters from Fisher Scientific, UK.

Lipase from *Rhizopus niveus* (Lipase RN, approximately 83 kDa, cat# 62310) and calcium chloride dehydrate (≥ 99.0%) were purchased from Sigma–Aldrich, UK. < 0.1% fat UHT milk and anhydrous milk fat were commercially purchased. Dialysis tubing cellulose membrane (MWCO 12000-14000, avg. flat width 25 mm (1.0 in.)) was purchased from Sigma-Aldrich, UK.

4.2.2. Instrumentation

Partition experiments were run in triplicate at 37 °C, using 50 mL Corning® PP self-standing centrifuge tubes, the USP 2 paddle apparatus (Agilent 708-DS Dissolution Apparatus) or 100 mL glass DURAN™ bottles (Fischer). All samples were analysed in an HPLC system consisting of an Agilent 1200 series binary pump (G1312A), an Agilent 1200 series DAD detector (G1315D), an Agilent 1200 series autosampler (G1329A), an Agilent 1200 series controller (G1316A) and Chemstation software (Agilent Technologies, Santa Clara, United States). A pH meter Mettler-Toledo AG (model SevenCompact pH/Ion S220, Schwerzenbach, Switzerland), a centrifuge Hereus Biofuge Primo R (Thermo Scientific, Hanau, Germany) and a vortex mixer Rotamixer (HTZ, Cheshire, UK) were used.

4.2.3. Model drugs selection

Assessment of the drug partition to fat was conducted for 15 drugs of a wide range of lipophilicity, ionisation, aqueous solubility, *and in vivo* food effect (Table 4.1). Working drug concentrations were selected according to experimental drug aqueous solubility values as reported in the literature or calculated values where experimental values were not available (Advanced Chemistry Development (ACD/Labs) Software v.11.02, Sci-finder), so as to avoid possible drug precipitation as the drug was initially dissolved in the "aqueous" (the part which does not contain fat, consisting of a mixture of skimmed milk and acetate buffer, $FeSSGF_{sk}$) of the fed-state medium used in the study. Due to its extremely low solubility in water (< 0.1 μg/ mL), working concentration of MK-C4 was selected according to its solubility in the "aqueous" phase of the medium in a 24 h period, performed using the shake-flask method [14]. In summary, an excess of drug was added to the solubility medium and left to equilibrate for 24 h at 37 °C under constant shaking. An aliquot of the saturated medium was initially filtered through a GF/D 2.7 μm filter and quantified after addition of ACN (2 parts in 1 part of medium), vortex (30 sec at full speed), centrifugation (15 min, 8000 rpm, 37 °C) and finally filtration through a regenerated cellulose filter. Drug was quantified in HPLC.

Table 4.1. Physicochemical properties and working concentrations of model drugs; "+" indicates positive food effect, "-"negative food effect and "n.e." no food effect.

Drug	Food effect [15-27]	log P [28-40]	log D $_{pH\ 5}$[a]	pKa [40]	Un-ionised fraction $_{pH = 5}$	Working concentration (µg/mL) [41-45]
Furosemide	-	0.74	0.90	4.25	3.07	80
Griseofulvin	+	2.18	2.51	-	100	8.6
Phenytoin	+	2.47	2.52	9.47	99.95	27
Ibuprofen	n.e	2.48	2.86	4.91	16.63	84
Indoprofen	n.e	2.86	1.86	3.74	16.20	128
Nifedipine	+	2.91	3.48	5.33	99/93	10
Propafenone hydrochloride	+	3.39	0.68	9.63	0.01	150[b]
Ketoconazole	-	3.72	2.47	6.75	3.35	2.7[b]
MK-C1[c]	n.e	4	4.11	6.5	99.75	3[c]
Danazol	+	4.20	3.35	-	100	1
Atorvastatin calcium	n.e	4.22	4.07	4.33	17.83	2.6[b]
Indomethacin	n.e	4.27	3.67	3.8	23.80	15
Felodipine	n.e	4.5	4.84	-	99.93	1.1
Itraconazole	+	6.20	5.09	3.70	22.82	3.7[c]
MK-C4[c]	+	8.81	9.06	-	100	3.2[d]

[a] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2016 ACD/Labs)

[b] Sci-Finder

[c] Physicochemical properties and food effect data provided by Merck

[d] Solubility study (24 h) performed in $FeSSGF_{sk}$

4.2.4. Drug partition to fat studies

A modified version of Fed State Simulated Gastric Fluid ($FeSSGF_{sk}$) was selected in order to simulate the "aqueous" phase of the working fed state medium. $FeSSGF_{sk}$ was prepared according to Jantratid et al. [46] by mixing skimmed milk and acetate buffer pH = 5 at a 1:1 volume ratio. For the preparation of 1 L of medium, 500 mL milk and 480 mL "blank"

acetate buffer were mixed under constant stirring using a magnetic stirrer. pH was adjusted to 5 with 1 N HCl and the volume was adjusted to 1 L with the "blank buffer". Anhydrous milk fat was selected as a surrogate of the lipid phase of FeSSGF. It is a cream or butter derivative, having water and proteins removed, and contains at least 99.8% milk fat [47].

Three different setups were developed for the assessment of drug partition rate from the aqueous to the lipid part of the fed gastric medium (Table 4.2). The three setups used 50 mL centrifuge tubes for diffusion through a dialysis membrane (setup I), the USP dissolution apparatus 2 (setup II) or 100 mL glass bottles for partition assessment of drugs in a smaller scale (setup III). For every drug, working solutions were prepared in $FeSSGF_{sk}$ and left under constant stirring at 37 °C for 60 min at concentrations equal to the drugs' aqueous solubility values. Appropriate quantities of anhydrous milk fat, equivalent to the desired w/v percentage of the total volume of each setup (pre-heated at 37 °C) (Table 4.2) were weighed. Appropriate volume of the $FeSSGF_{sk}$ drug solution was placed in the receptor vessel of each setup. A working temperature of 37 °C was maintained by the means of a 37 °C incubator room, a thermostated jacket or a heating plate for setups I, II and III respectively. Drug partition rate to fat was described by measuring the % decrease in the donor concentration with time, with samples taken from the middle of the $FeSSGF_{sk}$ layer at defined time points for a period of 24 or 48 hours. Experiments were performed in triplicate and % drug partition to fat was expressed as mean ± standard deviation. The exact quantities and volumes of lipid and aqueous parts, sampling time points and agitation conditions are stated in Table 4.2. The experimental setup suitability was evaluated with a pilot study of five drugs of different lipophilicity [propafenone hydrochloride, ketoconazole, nifedipine, danazol and atorvastatin calcium, ($\log D_{pH\ 5}$ = 0.68-4.07)].

The effect of fat percentage used was assessed in setup I (dialysis membrane setup), using nifedipine as the model drug. The quantity of fat placed in the membrane was equivalent to 5%, 8%, 15%, 20%, 25% w/v fat concentrations. 0.5 mL samples were collected from the donor compartment (30 mL of milk-based medium) at defined time-points (Table 4.2) and agitation was maintained by a 15 x 6 mm magnetic bar rotating at 300 rpm in a centrifuge tube with conical base and skirted bottom. Partition experiments using setup I were performed for the other four drugs of the pilot study using the 25% w/v fat concentration.

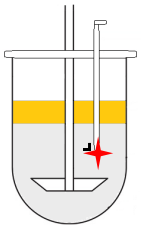
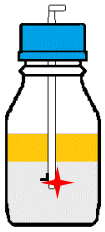
The effect of agitation speed in was evaluated in setup II, using the dissolution apparatus paddle at 3 different speeds (150, 200 and 250 rpm) and danazol as the model drug

and fat quantity equivalent to 25% w/v concentration of the total medium (Table 4.2). The volume of $FeSSGF_{sk}$ used was 500 mL. Agitation was stopped for 2 min for the two layers to separate before sampling. 5 mL samples were withdrawn at defined time-points for a duration of 8 hours and volume was replaced with drug solution in $FeSSGF_{sk}$. Partition experiments using setup II were performed for the other four drugs of the pilot study using 150 rpm agitation speed. Setup III, like setup II, was a biphasic setup without the presence of dialysis membrane, but performed at a smaller scale (30 mL of $FeSSGF_{sk}$). Agitation was provided by a 15 x 6 mm magnetic bar at 300 rpm and 0.5 mL samples were collected from the drug donor compartment at defined time-points for a period of 8 hours.

Once optimum parameters (fat percentage, medium volume, agitation means and speed) were selected, they were applied to all 15 drugs of the study. The experimental setup and fat percentage added for the final setup developed were selected on the basis of providing adequate discrimination among drug partition profiles and reasonable times for complete profiles.

Partition experiments in the presence of a surfactant were conducted using the partition setup III in order to assess the effect of emulsification conditions in the fed gastric environment on drug rate of partition. The partition rate was evaluated in the presence of an anionic surfactant (SLS) and also in the presence of a gastric lipase equivalent (RN lipase) dissolved in a $CaCl_2$ solution added at a concentration yielding activity similar to the physiological values (approximately 40 U/mL) [10, 48]. 6 mL of a stock solution of SLS or RN lipase in acetate buffer pH 5 was added to 24 mL of drug solution in $FeSSGF_{sk}$ (same drug concentrations as in partition "control" experiments without the presence of SLS or enzyme). $CaCl_2$ was added to a total 1.4 mM concentration. The concentration of the surfactant/enzyme stock solutions were selected so as to achieve a 1% w/v (for SLS) or 40 mg/mL (for lipase) concentration in the total volume of the system. $FeSSGF_{sk}$ and surfactant/lipase were left to mix for 5 min and the fat layer (10.3 g, Table 4.2) was added on top of the drug donor ($FeSSGF_{sk}$ and surfactant). 0.5 mL samples were taken from the drug donor at defined time-points for a period of 8 hours. Experiments were performed in triplicate and % drug partitioned was expressed as mean ± standard deviation.

Table 4.2. Experimental conditions of drug partition to fat setups.

Setup	I	II	III
Anhydrous milk fat $FeSSGF_{sk}$ Sampling point			
$FeSSGF_{sk}$ volume (donor) (mL)	30	500	30
Anhydrous milk fat nominal quantity (g)	10.3	172	10.3
Anhydrous milk fat nominal concentration (% w/v)	5-25	25	25
Sampling compartment	Centrifuge tube (50 mL)	Paddle apparatus vessel	Glass bottle (100 mL)
Sampling point	Middle of $FeSSGF_{sk}$ layer		
Sample volume (mL)	0.5	5	0.5
Agitation speed (rpm)	300	150	300
Membrane length (cm)	8 cm	-	-
Membrane type	Cellulose membrane avg. flat width 25 mm (1.0 in.), MWCO 14000	-	-
Sampling time points (h)	0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 24, 48*	0.17, 0.33, 0.5, 1, 1.5, 2, 3, 4, 6, 8	0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8
Temperature (°C)	37		

*24 h sampling time for nifedipine, 48h sampling time for all other drugs

4.2.5. Sample treatment and drug quantification

Drug quantification in $FeSSGF_{sk}$: 2 parts of methanol were added to 1 part of $FeSSGF_{sk}$ immediately after its sampling and the mixture was vortexed (30 sec), centrifuged (8000 rpm, 15 min, 4 °C), filtered through a 0.45 μm RC filter and analysed with HPLC. Drug was quantified against a set of calibration standards in $FeSSGF_{sk}$ treated as described above. Modifications of published chromatographic methods were used for drug quantification (Table 4.3).

Table 4.3. HPLC methods (or modification of published methods) used for the quantification of the model compounds.

Drug	Column	Mobile phase	Flow rate (ml/min)	Temperature (° C)	Inj. Vol. (µl)	λ (nm)
MK-C4*	Phenomenex Onyx monolithic C_{18}, 300Å, 250 x 4.6 mm, 5 µm	ACN:H_2O 70:30	3.5	40	100	220
Itraconazole [49]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 80:20	1	35	100	260
Felodipine [48]	Waters XBridge Shield RP18, 130Å, 150 x 4.6, 3.5 µm	ACN:H_2O 70:30	1	25	100	238
Indomethacin [50]	Waters XBridge Shield RP18, 130Å, 150 x 4.6, 3.5 µm	MeOH:Phosphoric acid 1.67% v/v	1	23	50	270
Atorvastatin calcium [51]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.025 M (pH 6) 40:60	1.5	30	100	246
Danazol [52]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 75:25	1	25	100	285
MK-C1*	Waters Symmetry Shield C_{18}, 100Å, 50 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.025 M (pH=2.5) Gradient (0-2 min 65:35/ 2-2.01 min 90:10/ 2.01-3 min 90:10/ 3-3.01 min 65:35	3	40	100	214
Ketoconazole [39]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O:DEA 75:25:0.1	1	25	100	260
Propafenone hydrochloride [53]	Agilent Eclipse XDB C_{18}, 120Å, 250 x 4.6 mm, 5 µm	MeOH:ACN: TEA:H_2O 50:7.5:0.1:q.s 100 (pH 2.9)	0.8	25	20	248
Nifedipine [54]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 60:40	1	20	50	238
Indoprofen [55]	Waters XBridge Shield RP18, 130Å, 150 x 4.6, 3.5 µm	ACN:Formic acid 0.1% v/v 40:60	1	40	50	280
Ibuprofen [56]	Agilent Eclipse XDB C_{18}, 120Å, 150 x 4.6 mm, 5 µm	MeOH:Acetic acid 0.2% v/v 60:40	1	25	100	233

Phenytoin [50]	Agilent Zorbax SB-C_{18}, 150 × 4.6 mm, 3.5 µm	ACN:H_2O 80:20	1	20	10	210
Griseofulvin [50]	Waters XBridge Shield RP18, 130Å, 150 x 4.6, 3.5 µm	MeOH:H_2O 65:35	0.8	20	50	292
Furosemide [57]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Formic acid 0.1% v/v 60:40	0.8	25	20	233

*HPLC methods provided by Merck

4.2.6. Data analysis

4.2.6.1. Data fitting

In order to determine the rates of drug partition, partition data (drug % partitioned vs time) were fitted to a first-order model (Eq. 4.1) using GraphPad Prism® v.7 software (GraphPad, US). Goodness of fits was assessed on the basis of coefficient of determination and normality test.

$$C_{part\ t} = C_{part\ max} * (1 - e^{-k_{part}t}) \quad \text{(Eq. 4.1)}$$

$C_{part\ t}$ is the % drug partitioned to fat at time t, $C_{part\ max}$ is the % maximum drug partitioned to fat and k_{part} is the first-order partition rate

Drug partition rate constants were compared using a two-way analysis of variance (ANOVA) repeated measures test for the different drugs and experimental setups, with Bonferroni's post-hoc test, using GraphPad Prism® v.7 software. The effect of surfactant/lipase was evaluated using a two-way ANOVA for the comparison of partition rates among different drugs and emulsification conditions with Bonferroni's post-hoc test. Statistical significance level was set at $p < 0.05$.

4.2.6.2. Multivariate data analysis [Partial Least Squares (PLS) regression]

The drug partition rate to fat was correlated to drug physicochemical properties [lipophilicity (log $D_{pH\ 5}$), aqueous solubility at pH 5 (μg/mL), molecular weight (MW)] and food effect observed in *in vivo* human studies by partial least squares (PLS) regression using the XLSTAT software (Microsoft, US). Interactions of $logD_{pH\ 5}$ with the above parameters (aqueous solubility at pH 5 (μg/mL), molecular weight (MW) and food effect) were also included in the model. The main advantage of PLS as a regression technique is the possibility to analyse data with independent variables which may be highly collinear [58]. The parameters were selected on the basis of the physicochemical aspects which control drug diffusion process between an aqueous and lipid layer. Assuming that the mechanism controlling the partition process is governed by the same basic principles as in lipid bylayers of biological membranes, drug partition is also dependent on drug diffusion coefficient and drug partition coeficient into the membrane barrier. Diffusion coefficient is dependent on size, shape and solvent-drug interaction. Therefore, even though log D is a good predictor for drug's partition rate to fat, other parameters such as drug size and molecular weigh, which corelate to the diffusion

coefficient, have to be considered. Interactions of log $D_{pH\ 5}$ with the rest of the properties were included in the model with an aim to elucidate possible drug partition mechanisms for which drug lipophilicity alone cannot account for.

The model quality was evaluated on the square of the coefficient of determination (R^2) and goodness of prediction (Q^2). R^2 and Q^2 values close to 1 refer to a model of good fit and prediction power respectively while a difference of R^2 and Q^2 lower than 0.2-0.3 between them is indicative of a successful model [59]. A Q^2 value > 0.5 was considered acceptable for good model predictability [60]. Full cross-validation (leave-one-out procedure) was used to develop and evaluate the regression model. The optimum number of calibration factors (principal components) for each model was selected based on the model's optimum predictability (Q^2) and predicted residual error sum of squares (PRESS). Lower PRESS values indicate better prediction [61], with the number of latent variables where PRESS starts increasing indicating the number of variables which to be retained in the model [62]. The importance of each parameter was evaluated by its variable importance in projection (VIP) value. Values above 1.0 are considered to have a significant effect on the dependent parameter, whereas values < 0.7-0.8 are not of statistical significance [59]. The standardised coefficients indicate the relative positive/negative effect of their corresponding parameter on the first-order rate of drug partition to fat (response value). High absolute values of standardised coefficients for variance X denote a big positive or negative effect on response Y. Outliers in the PLS model were evaluated on the basis of DModY (distance to model; residuals of Y), which express distance from each point to the PLS model with respect to the responses with high values.

4.3. Results and Discussion

4.3.1. Effect of fat percentage on drug rate of partition

The rate of drug partition to fat, was highly influenced by the percentage of fat present in the medium, as studied with partition setup I (dialysis membrane). A two-fold increase was observed in the rate of partition of nifedipine (drug with the highest fat affinity of the initial five compounds tested) when the fat content was increased from 5 to 25% w/v in the total medium (lipid and milk) volume (Figure 4.1 a). The rate of nifedipine partition was doubled when fat concentration increased from 5 (0.05 h^{-1}) to 25% w/v (0.1 h^{-1}) when partition data were fitted to a first-order equation, which can be attributed to the larger available area for diffusion when higher fat volumes are used, as described by Fick's first law of diffusion [63]. For the three higher percentages used though (15, 20 and 25% w/v), the rates lied

approximately between 0.08 and 0.1 h^{-1} (Figures 4.1 a, b). This signified a reduced effect of the fat percentage in partition rate for high fat medium content values. By increasing the amount of fat present in the receptor compartment, the percentage of total drug partitioned to fat in a period of 24 h also increased from 58% to 88% for 5% w/v and 25%w/v fat respectively. The pore size of the membrane (MWCO 14000), was multiple times higher than the molecular weight of the model drug of the study (nifedipine-346.33 g/mol), and therefore allowed the process to be controlled by the affinity of drug for the receptor rather than the membrane.

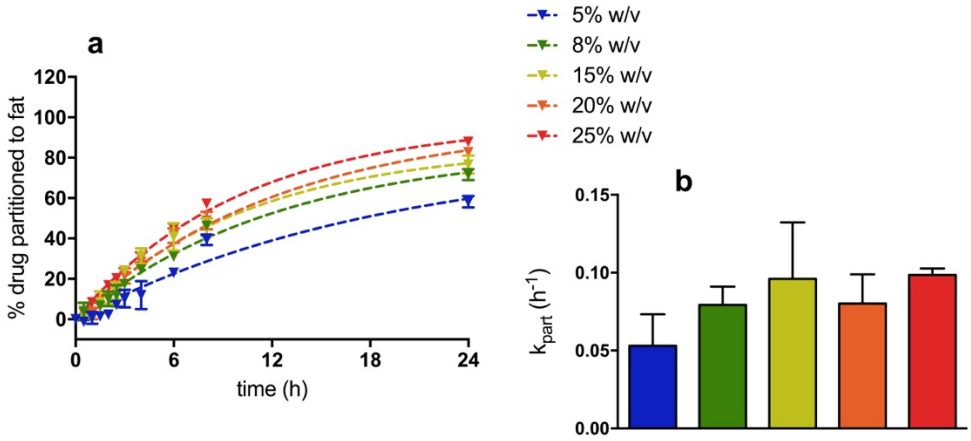


Figure 4.1. a. Nifedipine partition profiles to fat using setup I (dialysis membrane) and different fat percentages. Dashed lines denote the fittings to the first-order model. **b.** Bars denote the first-order partition rates of nifedipine partition to fat in setup I.

4.3.2. Effect of hydrodynamics on drug rate of partition

The evaluation of the effect of agitation in the drug partition setup II, using danazol as model drug for the study is presented in Figure 4.2. Using increased agitation rates (200 and 250 rpm), almost 100% of the drug was diffused in the lipid layer during the first 30 min. Such high agitation would be difficult to use with model drugs which partition to fat faster or equally fast as danazol, as it would possibly provide inadequate discrimination among them; It can be seen that the profiles in the two high agitation rates were very similar (Figure 4.2) and that the maximum portioned percentage is reached in the first 15 min. When the dissolution apparatus paddle was rotated at 150 rpm, a significantly slower partition profile was acquired with approximately 60% of the drug partitioned from the $FeSSGF_{sk}$ to the lipid compartment in the first hour, and 96% of the drug partitioned in the lipid layer in 8 h (Figure 4.2). Drug diffusion

to the lipid layer is regulated by two static diffusion layers developed in the two sides of the oil-"aqueous" interface with drug diffused through them from the aqueous to the lipid part [64]. Assuming that the width of the two layers remains constant though time, the parameters affecting the partition behaviour are: liquid viscosity, vessel dimensions, type of agitator and agitation speed, with the latter being the only parameter changing in the current study, justifying the differences observed in the rate of drug partition [64].

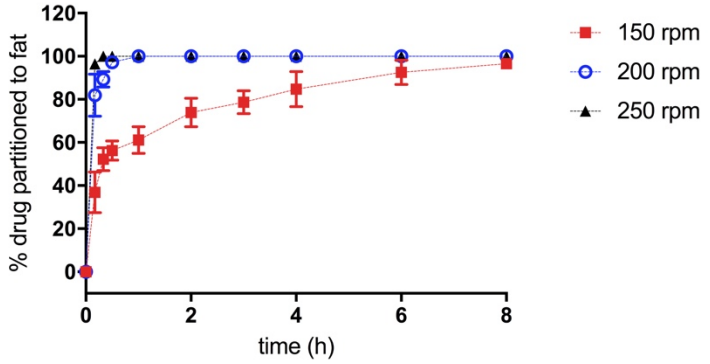


Figure 4.2. Danazol partition profiles to fat using the setup II under different agitation conditions.

4.3.3. Effect of experimental setup on drug rate of partition

The 25% w/v fat percentage was the percentage selected for the evaluation of the different proposed setups for the discrimination of partition rates to fat between drugs, even though the % w/v fat in the FDA high-fat standard breakfast [65] or in fed gastric media used *in vitro* such as milk, FeSSGF (Fed State Simulated Gastric Fluid) [46] and Ensure® Plus [66] is lower (≈ 1.8-14% w/v). The high fat percentage provided the highest partition rate compared to the other fat concentrations studied (Figure 4.1), making the discrimination among the various drugs easier, as observed from the results of the pilot study in setup I.

The five model drugs which were evaluated in the pilot study, using all three setups (Figure 4.3), all provided significantly different partition rates to fat ($p < 0.05$, two-way ANOVA) (Figure 4.4). In all setups, nifedipine showed the highest affinity for the lipid phase, while the whole amount of propafenone hydrochloride practically remained in the "aqueous" part throughout the duration of the study, possibly because of the latter's low distribution constant (log D = 0.68) in the working pH.

Using setup I, the whole process was extremely slow for the four of the five model compounds of the study, with the exception of nifedipine, with the amount of drug portioned to the fat being < 20% in the first 8 h (Figure 4.3 a). A plateau of the percentage partitioned could not be reached even after 48 h for all the model compounds, while phase separation of the milk-based medium was observed after 2 days. The decreased rate of the drugs' partition process can be attributed to the increased viscosity of the receptor (fat), which slowed down drug diffusion [67]. The slow reaction rates (especially for compounds other than nifedipine) may be considered a disadvantage for the current setup. Moreover, using above setup, no significant differences were observed ($p < 0.05$) among the rates of drug partition for the five model drugs used (Figure 4.4).

Setup II resulted in the highest partition rates for the five drugs initially studied (Figure 4.3 b). Discrimination among the partition rate of the model compounds to fat was observed (Figure 4.4). The drugs' partition rates to fat ranged between 0.39 h^{-1} (for ketoconazole) and 13.58 h^{-1} (for nifedipine). The volumes used were similar to the fed gastric volume *in vivo* [68, 69], but the hydrodynamics of this set up are different from the hydrodynamics observed in the fed stomach, as portrayed by the differences in Reynold's number between the fed stomach and the vessels in dissolution studies. USP 2 dissolution apparatus, at speeds between 50 and 100 rpm, results in Re numbers between 5000 and 10000 [70], while the equivalent values of the fed stomach *in vivo* are between 0.01 and 30 [71].

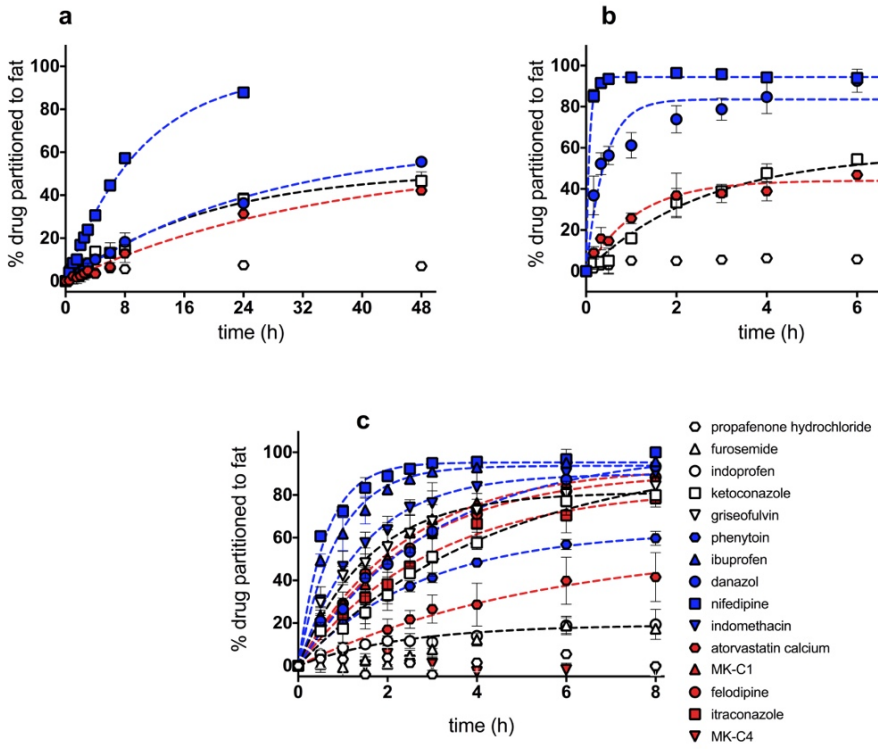


Figure 4.3. Drug partition profiles to fat using **a.** setup I, **b.** setup II and **c.** setup III. The marker colour is representative of drug lipophilicity (log $D_{pH\ 5}$); white colour for the five more hydrophilic drugs, blue colour for the five moderately lipophilic and red for the five most lipophilic. Dashed lines denote the fittings to the first-order model.

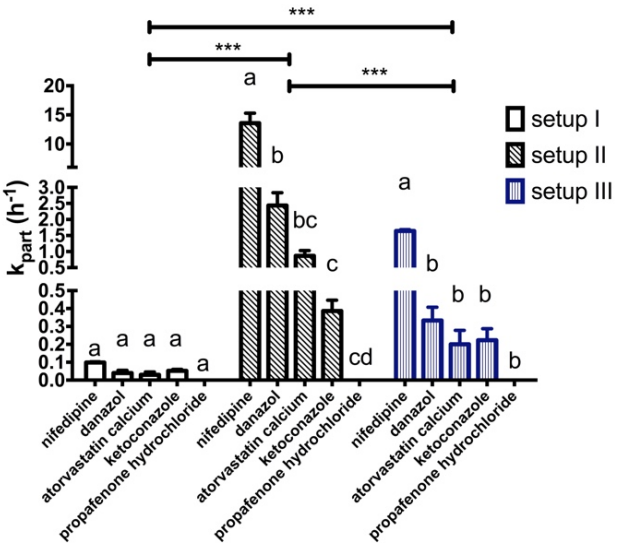


Figure 4.4. Calculated first-order rates of drug partition studies. Stars denote statistical differences among setups (two-way ANOVA, Bonferroni post-hoc test, * < 0.05, ** < 0.01, *** < 0.001). Different letters denote statistically significant differences ($p < 0.05$) among partition rates within the same setup.

Since the stationary level of the fluid is multiple times higher in the USP 2 dissolution apparatus, and is the factor with the biggest effect, (as the medium is the same in both cases),

it would be reasonable to assume that R_e values in the setup III model are probably closer to the values resulting from the hydrodynamics developped in the fed stomach *in vivo*. The partition rates to the fat for the drugs studied in setup III were: 0.22 (± 0.06), 0.20 (± 0.07), 0.33 (± 0.07) and 1.64 (± 0.04) h^{-1} for ketoconazole, atorvastatin calcium, danazol and nifedipine respectively, while propafenone's transfer to fat was insignificant (Figure 4.3). Adequate descrimination between the drugs' partition profiles, lower medium volume and drug consumption and reproducible results were obtained with this experimental set up (Figure 4.4). Therefore, it was selected for the investigation of partition to fat for the rest of the drugs. Setup III partition data were successfully fitted to the first-order equation model, with R^2 values of 0.90-1.00 and residuals randomly scattered (Figure 4.3, Table 4.4). The highest partition rates were observed for nifedipine (1.64 h^{-1}) and ibuprofen (1.17 h^{-1}), followed by indomethacin (0.70 h^{-1}) and griseofulvin (0.63 h^{-1}) (Table 4.4). It can be observed that the four drugs partitioned to the lipid part the fastest are of intermediate lipophilicity (log $D_{pH\ 5}$ = 1.86-3.67). Our hypothesis is that the increased partition rate of drugs of moderate lipophilicity is attributed to a combination of adequate drug affinity to fat and also high drug amount available in soluble form in the aqueous donor compartment. The above hypothesis is based on the principles which govern the incorporation of lipophilic drugs in previously formed liposomes, where despite drug increased drug lipophilicity, its rate of incorporation is controlled by the amount of drug available in the aqueous donor phase, with drug dissolution in it being the rate limiting step, often leading to very slow rates if not adequate [72]. The decreased drug solubility in the medium may explain the absence of drug partition to fat for MK-C4, the most lipophilic drug in the current study (Figure 4.3 c, Table 4.4). The rate of partition of the other four drugs of high lipophilicity (drug represented with red markers in partition profiles, Figure 4.3 c) ranged from approximately 0.2 to 0.4 h^{-1}. The absence of partition (rate ≅ 0 h^{-1}) for propafenone hydrochloride and furosemide (Figure 4.3, Table 4.4) was attributed to decreased drug lipophilicity (log D < 1) in the working pH. The percentage of the total drug partitioned to the fat in the duration of the study (8 h) was > 78% for 9 of the 15 model drugs (Figure 4.3 c, Table 4.4). The lower C_{8h} percentages were observed for the least lipophilic drugs of the study (propafenone hydrochloride, furosemide, phenytoin, indoprofen) can be attributed to their low affinity for fat. Drug transfer process between these two immiscible layers is governed by three steps; firstly, its diffusion towards the interface, its de- and re-solvation at the interface and, lastly, a new diffusion step from the interface to the lipid layer [73]. Therefore, the insignificant

partition of the drug with the highest log D, MK-C4, could possibly be explained by the drug's limited ability to dissolve in the "aqueous"/organic interface.

4.3.4. Effect of drug physicochemical properties on drug rate of partition

The relationship between drug partition rate and drug lipophilicity (log $D_{pH\ 5}$) follows a bell-shaped distribution around a maxium of log D = 3-4 (Figure 4.3 a). Several moderately lipophilic drugs (nifedipine, ibuprofen) of the study partitioned to fat faster than others of higher lipophilicity (felodipine, itraconazole) indicating that lipophilicity is not the sole parameter affecting the process.

Partition data (rates) showed that molecules of molecular weight higher than 500 g/mol partition to the lipid layer of the gastric medium at a slow rate relatively to the other model drugs, despite their high lipophilicity (e.g. atorvastatin calcium, ketoconazole, itraconazole, MK-C4) (Figure 4.5). Ionisation is also a parameter affecting drug partition into the lipid phase; for ionisable compounds, their un-ionised form is more easily partitioned to the lipid membranes [74]. It can be assumed that having the model drug in its un-ionised from in the aqueous donor ($FeSSGF_{sk}$) would facilitate its partition to the lipid layer. Out of the 15 model drugs of the study, the ones being ionised at a percentage higher than 95% in the working pH (according to their pKa values, Table 4.2) demonstrated the lowest partition rates, regardless of their lipophilicity (Tables 4.1, 4.4, Figure 4.5).

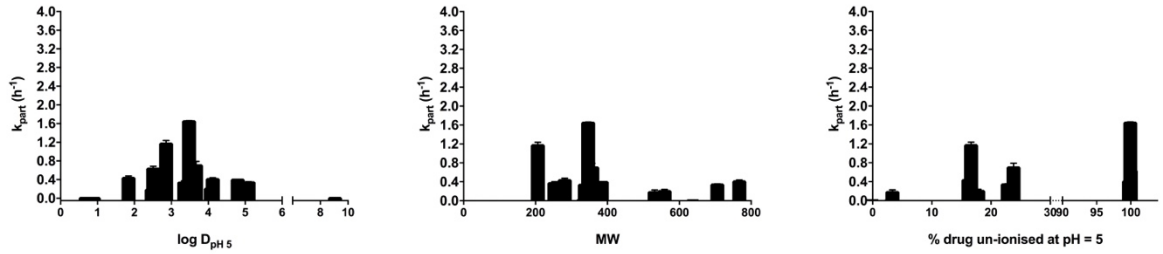


Figure 4.5. Drug partition rates to fat in the absence of SLS or RN lipase vs log $D_{pH\ 5}$, MW, and % of un-ionised drug in the working pH.

The impact of aqueous solubility at the working pH, lipophilicity, MW and *in vivo* food effect on drug partition behaviour was evaluated using partial least squares regression analysis. The initial PLS analysis for the rate of partition (1 principal component) which included all model drugs of the study, gave a model of moderate predictive power ($Q^2 = 0.35$) and fit ($R^2 = 0.44$) to the experimental values. Of the 15 drugs, nifedipine behaved as an outlier, with partition rates significantly higher than the model predicted (DMoDY values for nifedipine

were 2.2-2.4 times higher than the critical value given by the software for the specific PLS model). Reconstructing the model without including nifedipine gave a model defined by 1 principal component of Q^2 and R^2 values of 0.55 and 0.64 respectively, with predictive power acceptable according to the set threshold ($Q^2 > 0.5$).

Nifedipine's affinity to fat can be seen in the lipid percentage-dependent solubility of the drug in fed gastric media *in vitro*, as derived from its values in fed gastric media of 3.5, 1.75 and 0.875% w/v fat, (approximately 12x, 7x and 4x solubility for early, middle and late FeSSGF compared to aqueous buffers of the same pH and buffer capacity) [75] Fast partition to fat could be one of the reasons of nifedipine being a drug which exhibits positive food effect when administered with high fat meals [20] with more drug being solubilised by the lipid content of the gastric environment after meal administration.

Molecular weight and negative food effect were defined as negative predictors for drug partition to fat (negative standardised coefficients and high (> 1) VIP factors) (Figure 4.6). Molecular weight > 500 is considered a limiting negative factor for drug permeation to lipid membranes through passive diffusion [76, 77]. As observed by the negative standardised coefficient of the log $D_{pH\ 5}$*MW interaction, the effect of lipophilicity is different for drugs of different molecular weights. Looking at the drug partition rates (Table 4.4), it can be demonstrated that even extremely lipophilic drugs which do not have a reasonably low molecular weight, cross the lipid-aqueous interface barrier at a low rate (Figure 4.5). Positive log $D_{pH\ 5}$*aq $sol_{pH\ 5}$ interaction indicates that the effect of drug lipophilicity on the rate of partition to fat differs according to drug aqueous solubility. Positive food effect is generally associated with drug lipophilicity though lipid emulsification of lipophilic drugs in the stomach [78] and increase in drug luminal solubility [4]. The model build failed to demonstrate any correlation between positive food effect and drug-fat interaction, as the statistically non-significant negative coefficient indicates (Figure 4.6). On the contrary, the model demonstrated a negative correlation (Figure 4.6) between partition rate to fat with negative food effect *in vivo*. Drugs of which pharmacokinetic behaviour does not change as a result of meal administration (no food effect), appear to partition to fat significantly faster than the rest (highest positive standardised coefficient), which is another indication that partition to fat alone cannot be used as a sole predictor for changes in drug pharmacokinetic parameters after administration of high-fat meals.

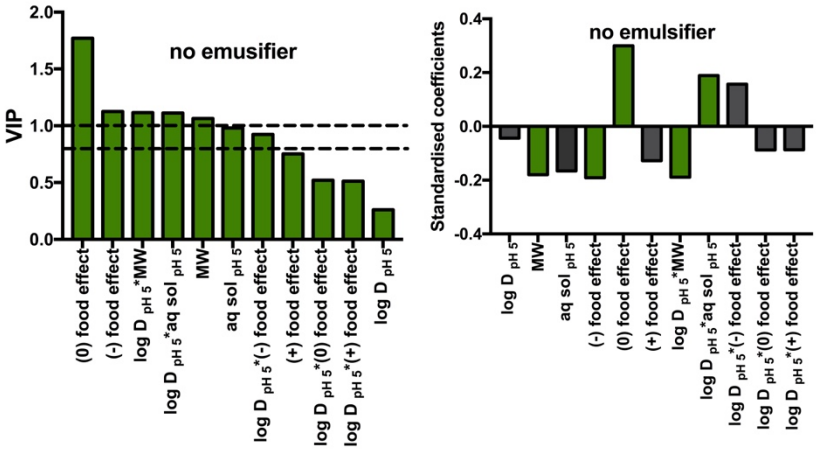


Figure 4.6. Variable importance in the projection (VIP) plot with the variables classed according to their importance of the response for drug partition rate (in the absence of enzymes or emulsifiers) to fat (left). Standardised coefficients corresponding to the variables (and their interactions) studied (right). Green colour denotes coefficients of VIP values > 1.

4.3.5. Effect of emulsification conditions on drug rate of partition

When 1% w/v of SLS was added to the drug donor, in order to simulate an extreme version of the emulsification of the fat taking place in the fed gastric environment, enhancement of the drug partition rate to fat was observed for 9/15 drugs following first-order kinetics both the presence and absence of surfactant). The increase in the rate of the partition process ranged from 11.4% (indoprofen) to 335.8% (felodipine), while a slight decrease compared to the partition rate in the absence of SLS was reported for three of the drugs (4.7, 10.0 and 29.5% decrease in partition rates for ibuprofen, griseofulvin and itraconazole respectively) (Figure 4.7, Table 4.4).

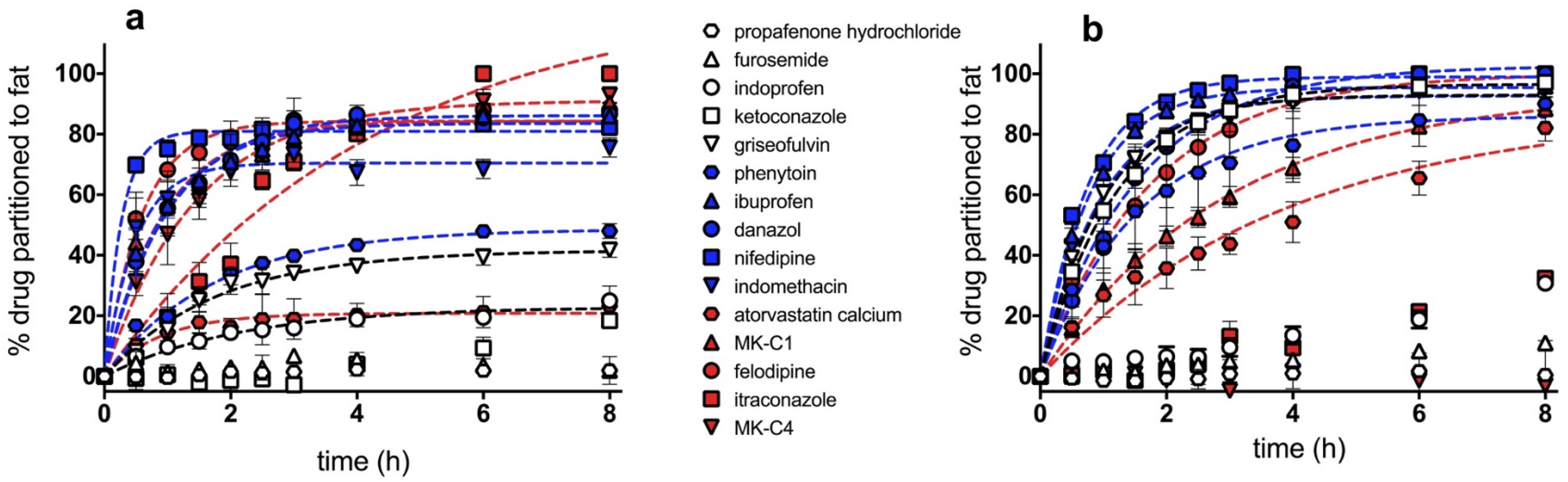


Figure 4.7. Drug partition profiles to fat using the setup III in the presence of **a.** SLS or **b.** lipase. The marker colour is representative of drug lipophilicity (log $D_{pH\,5}$); white colour for the five more hydrophilic drugs, blue colour for the five moderately lipophilic and red for the five most lipophilic. Dashed lines denote the fittings to the first-order model.

The presence of SLS in the drug donor compartment had a statistically significant effect on the drug partition rate, compared to the profiles in the absence of surfactant ($p < 0.05$). The difference in partition rates to fat is a result of two conflicting phenomena; i. higher drug affinity for the donor in the presence of surfactant and ii. bigger available receptor surface area for partition compared to the control experiment. The addition of SLS under constant stirring breaks the fat into smaller droplets which increases their surface area [79]. In the fed stomach, where fat emulsification takes place, as a result of the agitation conditions and the presence of lipid digestion products, the diameter of fat droplets is significantly reduced with a the emulsion surface area demonstrating a three-fold increase [2], which justifies the increase in partition rate in this study, after the addition of SLS. The presence of surfactants (emulsifiers, proteins or lipolysis products) in the lipid-water interface can reduce the surface tension increasing the diffusion kinetics by increasing the drug interfacial permeability, compared to the large surface tension of the non-emulsified lipid-water interface, where partition phenomena are slow [13].

The partition process is drug dependent with significant difference observed among different drugs ($p < 0.05$). Higher rates of partition to fat were observed for moderately lipophilic drugs (logD $_{pH\ 5}$ values 3-4) and lower rates for drugs of extreme low or high lipophilicity (Figure 4.8). A bell-shaped curve is observed for the correlation of partition rate with log D $_{pH\ 5}$ (Figure 4.8).

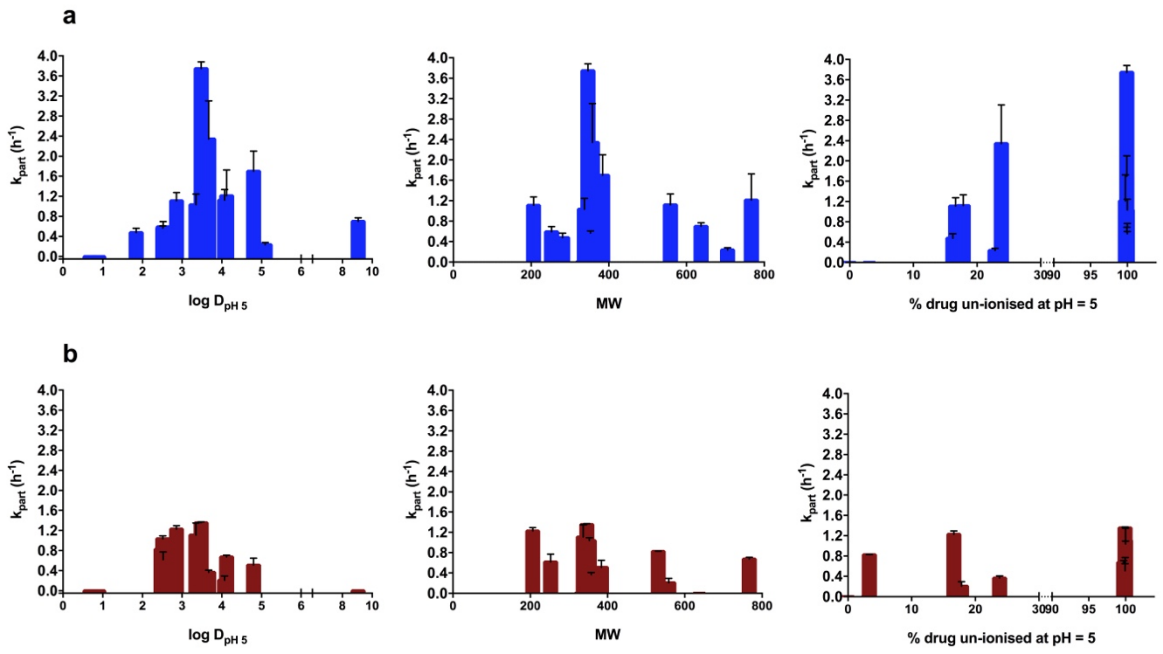


Figure 4.8. Drug partition rates to fat **a.** in the presence of SLS or **b.** RN lipase, vs log D $_{pH\ 5}$, MW and % un-ionised drug at pH 5.

The PLS analysis for the rate of partition (1 principal component) which included all model drugs of the study except for nifedipine, gave a model of good predictive power (Q^2 = 0.54) and fit (R^2 = 0.60) to the experimental values. Absence of food effect and interactions of log $D_{pH\ 5}$ with negative food effect and absence of food effect were defined as positive predictors for drug partition to fat (positive standardised coefficients and high (> 1) VIP factors) (Figure 4.9). Faster partition rates to fat both the presence and absence of the surfactant, in drugs of similar bioavailability in fasted and fed conditions, is another indication that drug rate partition to fat is not directly associated with *in vivo* positive food effect. On the contrary, a negative correlation with positive *in vivo* food effect can only be observed in the negative standardised coefficients in all three conditions (absence and presence of SLS or lipase) in the medium, which is only statistically significant in the presence of the ionic surfactant (Figure 4.9). In the fed stomach, during gastric emptying, aqueous content is transferred to the duodenum significantly faster than fat, which is held by the angular notch [2]. Therefore, if the drug is adequately soluble in the watery portion of the gastric content, aided by the natural surfactants present (a role played by the surfactant in the developed *in vitro* partition setup), slower partition to the lipid layer would mean increased drug quantity available for transfer to the intestinal environment. A slower partition to fat therefore, provided that the drug is adequately soluble and does not precipitate in the aqueous part of the stomach, may be associated with positive food effect. Negative effect of drug aqueous solubility is attributed to the high affinity or highly soluble drugs for the "aqueous" compartment.

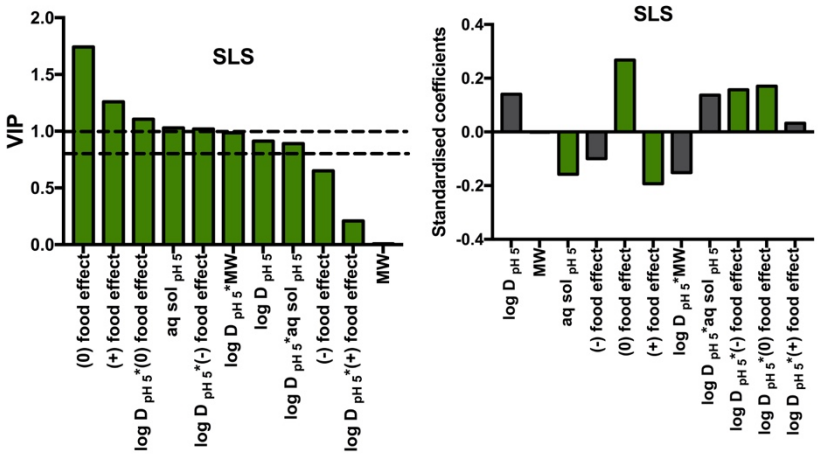


Figure 4.9. Variable importance in the projection (VIP) plot with the variables classed according to their importance of the response for drug partition rate (in the presence of SLS) to fat (left). Standardised coefficients corresponding to the variables (and their interactions) studied (right). Green colour denotes coefficients of VIP values > 1.

In the presence of the lipase, the differences in partition behaviour among drugs of different lipophilicity were subtler (Figure 4.8) than in the presence of SLS. Rates of partition ranged from 0.207 h^{-1} (atorvastatin calcium) to 1.354 h^{-1} (nifedipine) (Table 4.4). Although partition rates for all drugs were equally high or slightly higher than in the absence of the enzyme (Table 4.4, Figures 4.3 c, 4.7 b), the differences were not statistically significant, which implied that the partition rates to fat were not affected by the presence of the lypolytic enzyme. MK-C1 and nifedipine demonstrated slightly slower rates than in the absence of lipase by 1.11 and 1.21 times respectively. Similarly to the other two conditions (absence of surfactant/enzyme and presence of SLS), drugs of intermediate lipophilicity (blue markers in Figure 4.7 b) partition to fat faster and to a higher percentage than extremely lipophilic (red markers) and hydrophilic (white markers) drugs. Except for the three most hydrophilic drugs (propafenone hydrochloride, furosemide, indoprofen) and the extremely lipophilic MK-C4, a percentage higher than 80% of all drugs partitioned to the fat layer in a period of 8 hours except for itraconazole (Figure 4,7 b, Table 4.4). Interestingly, in the presence of lipase, itraconazole started diffusing to fat only after 3 hours and only 32.4% of the initial drug concentration partitioned to fat in 8 hours.

The bell-shaped distribution around log D values of 3-4, (Figure 4.8) indicates that in the presence of lipase, drug partition behaviour is not only governed by the drug lipophilicity, but it can be also controlled by other physicochemical parameters, an effect confirmed by the multivariate analysis of data. Looking at the partition rates against MW and un-ionised drug fraction plots (Figure 4.8), the rate of partition seems to be affected by MW and drug ionisation to a smaller extent.

The PLS analysis for the rate of partition (1 principal component) which included all model drugs of the study except for nifedipine, gave a model of good predictive power (Q^2 = 0.53) and fit (R^2 = 0.65) to the experimental values. Regression analysis demonstrated that the variables affecting drug partition behaviour remained almost unchanged in the presence or absence of gastric lipase. In both cases, the main variables with a negative effect on drug rate of partition were drug MW, $logD_{pH\ 5}$*MW interaction and drug aqueous solubility (Figure 4.10). The negative effect of the former is attributed to the negative correlation of molecular weight on drug diffusion coefficient [80]. The effect of $logD_{pH\ 5}$ differs with MW, as negative log D*MW interaction indicates. Drug aqueous solubility is negatively correlated with drug partition rate due to higher affinity for water-soluble compounds for the donor compartment. The positive standardised coefficient of $logD_{pH\ 5}$*$aq\ sol_{pH\ 5}$ indicates that a possible positive

correlation of drug partition rate with its lipophilicity is dependent on drug aqueous solubility. This positive coefficient, could be an indication that lipophilicity is a parameter favourably affecting the partition process, provided that the drug is adequately soluble in the donor compartment.

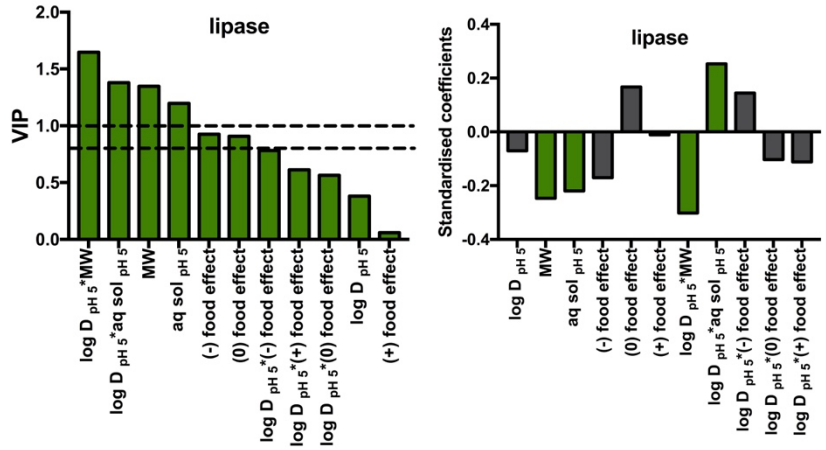


Figure 4.10 Variable importance in the projection (VIP) plot with the variables classed according to their importance of the response for drug partition rate (in the presence of lipase) to fat (left). Standardised coefficients corresponding to the variables (and their interactions) studied (right). Green colour denotes coefficients of VIP values > 1.

Table 4.4. Drug first-order partition rates to fat in the absence and presence of SLS and lipase.

Drug	No emulsifier		SLS		Lipase	
	k_{part} (h^{-1})	C_{8h}	k_{part} (h^{-1})	C_{8h}	k_{part} (h^{-1})	C_{8h}
propafenone hydrochloride	0 (0)*	-	0 (0)	-	0 (0)*	-
furosemide	0 (0)*	-	0 (0)	-	0 (0)*	-
indoprofen	0.429 (0.049)	19.4 (7.1)	0.477 (0.087)	25.0 (0.6)	N/A	30.8 (1.9)
ketoconazole	0.223 (0.037)	80.0 (1.2)	N/A	18.4 (1.8)	0.825 (0.002)	97.2 (0.8)
griseofulvin	0.628 (0.059)	84.1 (3.6)	0.565 (0.045)	41.7 (2.4)	1.036 (0.058)	95.5 (3.3)
phenytoin	0.364 (0.039)	59.7 (3.3)	0.592 (0.103)	48.0 (2.4)	0.617 (0.155)	90.1 (2.4)
ibuprofen	1.165 (0.073)	95.8 (0.5)	1.111 (0.163)	86.0 (1.0)	1.228 (0.065)	95.9 (0.46)
danazol	0.330 (0.043)	93.5 (0.9)	1.033 (0.213)	86.7 (1.8)	0.673 (0.036)	100 (0)
nifedipine	1.642 (0.022)	100 (0)	3.748 (0.134)	82.3 (1.2)	1.354 (0.018)	100 (0)
indomethacin	0.697 (0.095)	90.4 (1.7)	2.344 (0.760)	75.6 (3.2)	1.109 (0.240)	95.4 (2.1)
atorvastatin calcium	0.198 (0.043)	41.6 (11.5)	1.122 (0.211)	23.2 (6.6)	0.207 (0.087)	82.1 (4.3)
MK-C1	0.404 (0.036)	88.5 (14.1)	1.212 (0.513)	90.4 (1.0)	0.365 (0.044)	88.5 (1.7)
felodipine	0.390 (0.005)	88.4 (0.8)	1.699 (0.406)	87.9 (2.7)	0.673 (0.036)	98.9 (2.0)
itraconazole	0.335(0.020)	78.6 (2.8)	0.236 (0.044)	100 (0)	N/A	32.4 (2.5)
MK-C4	0 (0)*	-	0.703 (0.066)	93.0 (2.8)	0 (0)*	-

*partition was insignificant; partition rates were considered zero

N/A: the first-order model did not fit to the partition data

4.4. Conclusion

Drug interaction with fat has been closely related to possible *in vivo* food effect after drug administration with meals of high lipid content. The current study strove to depict the drug partition process to the lipid phase of the fed stomach content by developing an *in vitro* discriminating method, able to assess the differences in rates of partition to fat, of drugs of different physicochemical characteristics. Using model drugs of a wide range of lipophilicity, ionisation and food effect, the *in vitro* setup developed provided discrimination of drug partition rates. The study revealed that percentage of fat, lipid and aqueous compartment volumes and agitation conditions affect the rate of partition significantly. Simulations using a biorelevant gastric medium as a surrogate of the fed stomach content, reflected the dependence of the rate of partition not only to drug lipophilicity but also to other physicochemical properties such as its MW and aqueous solubility. Furthermore, the current investigation revealed a correlation between drugs demonstrating negative food effect and slow partition to fat. However, it is important to highlight that food effect on bioavailability depends on a number of complex mechanisms and cannot be predicted solely based on the elucidation of lipid-drug interactions in the fed stomach. Moreover, conclusions on drug partition rates must not only be based on API properties but the effect of drug formulation must also be considered. The effect of food texture (homogeneous meal vs meal with solid food components) is an aspect which can potentially affect the time available for drug to partition to the indigested fat and needs to be investigated in future studies. After administration of solid meals, fluids can be emptied from the fundus to the pyloric antrum and into the intestine with rates as fast as in the fasted state, around the caloric gastric contents along the stomach walls [81]. Therefore, the time for which drug is available to partition to the undigested lipid layer before being emptied with the watery phase in the small intestine needs to be taken into consideration. Conclusions on drug partition rates must not only be based on API properties but the effect of drug formulation must also be considered. The effect of drug surface activity on partition behaviour was not assessed in this work and should be considered in future studies. Several drugs of different physicochemical properties have been found to be surface active. Cases of drugs such as phenothiazines, antihistamines and local anaesthetics have been extensively reported in the literature [82-84]. Surface activity of several compounds has been associated with their affinity to lipid membranes [85, 86]. Demonstrating a detergent-like behaviour, drugs have the ability to self-associate and bind, disrupt and solubilize lipid bilayers [87]. Further studies, investigating also the drug “release” from the lipid part of the gastric fed content to the

intestinal environment combining the partition principles of developed setup with simulated intestinal media could potentially shed light towards better understanding of food effect mechanisms.

4.5. References

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Chapter 5: Towards the development of a biorelevant medium to simulate the gastric conditions in the fed state

Abstract

Fed state simulated gastric fluid (FeSSGF), a milk-based medium, has been used in biorelevant dissolution studies, simulating the fed state gastric environment. For drug quantification in heterogeneous media like FeSSGF, a laborious extraction technique prior to drug analysis is usually required. The aim of the current study was to develop a novel biorelevant medium with similar physicochemical properties to FeSSGF which would require a less time/labour consuming extraction protocol. A stepwise development approach was followed, gradually incorporating casein and a mixture of lecithin/triolein in the medium, to simulate drug solubilisation in casein micelles and polar/non-polar lipids of milk respectively. pH, osmolality, buffer capacity and surface tension of the non-milk-based developed medium ($FeSSGF_{nm}$) (were similar to the reference medium (FeSSGF). To evaluate its similarity to FeSSGF in terms of drug solubilisation and determine the effect of protein and lipid content in drug solubility, 24 h-solubility studies for 5 drugs of a range of lipophilicity (log P = 0.74-6.2) and ionisation (two weak bases, one weak acid and two neutral compounds) were performed in fed gastric biorelevant media of different lipid and protein content. $FeSSGF_{nm}$ overestimated drug solubility from 1.38 to 142 times, an effect more profound for the drugs of higher lipophilicity. Excluding the lipid part from the medium resulted in solubility values down to 20 times lower than in FeSSGF and inversely proportional to drug lipophilicity (log P). The study demonstrated that changes in lipid and protein content in the medium can be critical for drug solubility. To develop a FeSSGF-like biorelevant medium, further insight is required in simulation of drug solubilisation in casein micelles and its distribution to lipid components in terms of control of the size and composition of the lipid-protein aggregates.

Keywords: Gastric conditions, Biorelevant, Dissolution, Solubility, Fed state, Casein micelle

5.1. Introduction

It has been widely reported that meal intake can affect the pharmacokinetic parameters of multiple drugs through changes in the physiology of the gastrointestinal environment [1] and the physicochemical properties of its contents, such as the pH, osmolality, buffer capacity and surface tension [2-4]. Presence of bile salts [5], gastric secretions [2, 6] and fat content [7] are also parameters affecting drug bioavailability and can potentially be included to dissolution media designed to simulate the *in vivo* conditions in this state.

To date, several media have been proposed for the simulation of the gastric environment after administration of a meal, taking the above aspects into consideration [8]. The development of these more "biorelevant" media in the last two decades is the response to the need of mimicking the GI tract conditions with dissolution media able to be used for predictive dissolution testing, which is not often feasible with conventional compendial buffers. The long-term purpose of the development of such media is the correlation of *in vivo* drug release with the drugs' *in vivo* pharmacokinetic profiles (IVIVC) towards the reduction of the number of bioequivalent studies needed prior to their approval [9].

For the simulation of fed gastric content most *in vitro* developed media use versions of the FDA high-fat standard breakfast as points of reference for medium development, as food effect is more likely to be observed after administration of meals of high fat content compared to meals of lower fat percentage [10]. In general, such meals would have a 800-1000 kcal energy content, with approximately 500-600, 250 and 150 kcal deriving from fat, carbohydrates and proteins respectively [10]. Milk was one of the first fed state environment-surrogates used in dissolution studies, due to its simplicity and stability, despite being of lower energy content compared to a standardised meal administered in food effect studies [11, 12]. Dissolution studies in milk demonstrated significant differences in drug dissolution profiles compared to simple buffers [13]. It has been shown though, that milk osmolality and buffer capacity values are significantly lower compared to the equivalent values of homogenised high (62% kcal derived from fat) and low fat (37% kcal derived from fat) standard breakfasts [14], which indicates its limited biorelevance. Further studies suggested the presence of combinations of HCl, pepsin and lipolysis enzymes in the medium in order to account for lipid and protein digestion processes taking place *in vivo* [15, 16]). Other approaches included the use of more biolelevant media of high lipid content (up to 30% w/v), such as lipid emulsions normally used for parenteral administration (such as Ensure® Plus or Intralipid®) [17, 18].

One of the media lately suggested to simulate the gastric fed state environment is the Fed State Simulated Gastric Fluid (FeSSGF), developed by Jantratid et al. [19] approximately ten years ago. The three versions of the medium developed ($FeSSGF_{early}$, $FeFFGF_{middle}$ and $FeSSGF_{late}$) are composed of different ratios of full fat milk and acetate or phosphate buffers and simulate the gastric contents at the early (0-75 min), middle (75-165 min) and late (after 165 min) phases of the postprandial stomach. $FeSSGF_{middle}$ has been extensively used as a compromise for the simulation of the sum of the conditions of the gastric environment after meal administration. It has been successfully implemented in biorelevant dissolution for tablets and capsules of poorly soluble drugs (cinarrizine, nelfinavir, BCS IV weak base) achieving good predictions of plasma profiles in the fed state in combination with biorelevant intestinal media and coupled with pharmacokinetic simulation modelling [20-22] and revealing the advantages in dissolution using biorelevant media compared to compendial ones.

The challenge of the use of FeSSGF is its treatment, which due to its protein content requires techniques like protein precipitation prior to drug analysis [8]. To address the above issue, alternative versions of the three FeSSGF (early, middle and late) "snapshot" media were developed (FeSSGFEm) [23, 24], containing the equivalent amount of fat, deriving from a lipid emulsion (Lipofundin® MCT 20%) which did not contain any proteins. Analysis with simple filtration was possible for the above medium, but its resemblance in terms of drug solubilisation with the original version of FeSSGF remains in question, as lipophilic drugs are solubilised in milk via two mechanisms; i. solubilisation in the lipid part of the emulsion and ii. solubilisation in the casein micelles [25-27]. Milk is a not a typical oil-in-water emulsion and lipids in milk are organised in assembles in the form of fat globules, each constituting of a triglyceride-, cholesterol- and retinol ester core, emulsified by a tri-layered amphiphilic membrane (milk fat globule membrane, MFGM) of phospholipids, proteins and cholesterol [28, 29]. The most abundant proteins in bovine milk are caseins (approximately 80% of total milk proteins). Casein micelles are clusters of protein (50-600 nm) formed in milk and consist of four different casein types, αs1, αs2, β, and κ in the form of phosphate and calcium ions, which are in equilibrium state with their equivalent monomers [30, 31].

The aim of the current study was to develop a novel non-milk-based fed state gastric biorelevant dissolution medium with similar physicochemical properties and drug solubility/dissolution characteristics to FeSSGF, which would require an easier and less laborious sample treatment prior to analysis. Drug solubilisation in milky part of FeSSGF, in the two main drug solubilisation sites; proteins and fat globules was simulated in the

development of the new fed gastric medium. Triolein and egg lecithin were used to simulate the triglyceride core and membrane of the milk's fat globule respectively, whereas sodium caseinate (NaCas) was added in order to reproduce drug solubilisation in milk's casein micelles. A stepwise development of the final medium was performed with the goal to assess the role of the buffer, casein content and triolein in the medium's physicochemical properties and solubilisation power in drugs of a broad range of lipophilicity (log P = 0.74-6.20) and ionisation (two weak bases, one weak acid, two neutral compounds).

5.2. Materials and Methods

5.2.1. Materials

Furosemide (> 98% (HPLC), danazol (≥ 98%), itraconazole (≥ 98% (TLC)) and casein sodium salt from bovine milk (C8654) were all purchased from Sigma-Aldrich, UK. Nifedipine 98-102% (on dried substance)), glycerol (≥ 99%), sodium chloride, sodium hydroxide, sodium acetate trihydrate, hydrochloric acid 37% and glacial acetic acid ≥ 99% were all purchased from Fisher Scientific, UK and griseofulvin (> 97%) from Alfa Aesar, UK. Egg-lecithin (Lipoid E PCS, Phosphatidylcholine from egg) was acquired from Lipoid GmbH (Ludwigshafen, Germany) and 3.6% fat UHT-treated milk was commercially purchased (Sainsbury's, UK). All organic solvents used in protein precipitation and drug analysis were HPLC-grade and were purchased from VWR, UK.

Cronus 13 mm regenerated cellulose (RC) syringe filters 0.45 μm were purchased from LabHut Ltd, UK and Whatman 13 mm glass microfiber syringe filters 2.7 μm (GF/D) from Fisher Scientific, UK.

5.2.2. Instrumentation

All samples were analysed in an HPLC system consisting of an Agilent 1200 series binary pump (G1312A), an Agilent 1200 series DAD detector (G1315D), an Agilent 1200 series autosampler (G1329A), an Agilent 1200 series controller (G1316A) and a Chemstation software (Agilent Technologies, Santa Clara, United States).

A pH meter Mettler-Toledo AG (model SevenCompact pH/Ion S220, Schwerzenbach, Switzerland), a centrifuge Hereus Biofuge Primo R (Thermo Scientific, Hanau, Germany) and a vortex mixer Rotamixer (HTZ, Cheshire, UK) were used. A ring tensiometer (Sigma 700 Force tensiometer, Attension, UK) and an Advance Instruments Inc. microosmometer (Norwood, MA, United States) were used for the surface tension and osmolality measurements.

Organic solvent was evaporated using a rotary evaporator consisting of a Büchi Waterbath B-480 and a Büchi Rotovapor R-114 (Büchi Labotechnik, Flawil, Switzerland) was used for organic solvent evaporation.

5.2.3. Media composition

FeSSGF was developed according to the *in vivo* physicochemical properties measured in the fed gastric environment during the middle phase of the gastric digestion and has been fully characterized in terms of its physicochemical properties [19]. Medium pH, buffer capacity, osmolality, surface tension and lipid/protein content were used as the basis of the new medium development [19] and were selected on the basis of physicochemical properties measured *in vivo*, from samples taken from the stomach antrum of healthy volunteers after administration of Ensure® Plus [2]. Both FeSSGF and $FeSSGF_{nm}$ were designed to simulate the "middle" postprandial conditions (75-165 min after food administration).

5.2.3.1. Fed State Simulated Gastric Fluid (FeSSGF) composition

Fed State Simulated Gastric Fluid (FeSSGF) was prepared according to Jantratid et al. [19] by mixing 3.6% fat milk and acetate buffer pH = 5 at a 1:1 volume ratio. For the preparation of 1 L of medium, 500 mL milk and 480 mL buffer were mixed under constant stirring using a magnetic stirrer. pH was adjusted to 5 with 1 N HCl and the volume was adjusted to 1 L with the buffer. Medium composition is described in Table 5.1.

5.2.3.2. Non-milk-based Fed State Simulated Gastric Fluid ($FeSSGF_{nm}$) composition

Sodium caseinate concentration in the medium was set at 12.5 mg/mL, based on casein content experimental values reported for cow milk in the literature. Concentration of casein in milk lies between 24 and 32 mg/mL, depending on the bovine species, area and diet [32-34]; therefore a 25 mg/mL solution simulated the "milky" half part of the medium. Sodium caseinate was selected due to its high solubility (approximately 50 mg/mL in water according to the supplier) and on the basis that at pH 5, in the presence of 100 mM NaCl, sodium caseinate concentrations of 5% w/w form aggregates of approximately 150 nm size [35], similar to the casein micelle size formed in milk.

Milk fat consists mainly of triglycerides (98% w/w) of more than 400 different saturated and unsaturated fatty acids, with tripalmitin (16:0) and triolein (18:1), being the most prevalent [36]. Milk fat globules have the form of a triglyceride core, surrounded by a tri-layer of polar lipids and membrane proteins. The lipid part of the membrane is mainly comprised of

phospholipids and secondarily of cerebrosides and cholesterol [36]. Approximately 60-70% of the membrane's polar lipids consists of glycerophospholipids and sphingolipids [(mainly phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [37]], which represent 0.5%–1% of milk fat [38]. 1.8% w/v total amount of fat was used for the simulation of the lipid part of FeSSGF, with 99% of the total fat comprising of triolein and 1% of lecithin (egg phosphatidylcholine, E-PC).

$FeSSGF_{nm}$ was developed in steps with its physiochemical characteristics measured in each level of development. The rationale behind this design was the assessment of the role of proteins and lipids in drug solubility. The composition of each level is described in Table 5.1.

To prepare 1 L medium the following procedure was used: The same procedure was used for all development levels of the novel medium, with the gradual incorporation of ions (Level 1), various protein concentrations (Levels 2a-d), and lipid concentrations (Levels 3a-b) (Table 5.1). Level 3b represents the final developed medium ($FeSSGF_{nm}$).

Approximately 480 mL of "blank" buffer (Level 1) was prepared adding the amounts of NaCl, CH_3COOH, CH_3COONa and glycerine calculated for 1 L and transferred to a 1 L round-bottom flask. Glycerol was added as a co-solvent so as to achieve a more effective emulsification of the non-polar lipids added. Its concentration was selected on the basis of lipid/glycerol ratio of Lipofundin® MCT 20 (a lipid emulsion which has been used as a lipid part surrogate in biorelevant fed gastric media [23]). Appropriate volume of a lecithin solution (100 mg/mL in CH_2Cl_2) was added, with the organic solvent being evaporated under vacuum. The pressure was decreased from 650 mbar to 100 mbar in 2 min steps for a 15 minute-period and was subsequently maintained at 100 mbar for another 15 min (40 °C). Appropriate volume of a triolein solution (50 mg/mL in CH_2Cl_2) was added under the same conditions as lecithin. 500 mL of a second solution of NaCas in water (25 mg/mL) was prepared by continuous stirring. The two solutions were combined by vigorous stirring for approximately 2 h and the final pH was adjusted with 0.1 N HCl/NaOH. The final volume was adjusted with "blank" buffer.

Table 5.1. Composition of the development levels of $FeSSGF_{nm}$.

	FeSSGF	Level 1	Level 2a	Level 2b	Level 2c	Level 2d	Level 3a	Level 3b ($FeSSGF_{nm}$)
		Buffer	Buffer + proteins				Buffer + proteins + lipids	
NaCl (mM)	237.02	176.57	176.57	176.57	176.57	176.57	176.57	176.57
Sodium acetate (mM)	17.12	17.12	17.12	17.12	17.12	17.12	17.12	17.12
Acetic acid (mM)	29.75	29.75	29.75	29.75	29.75	29.75	29.75	29.75
Milk:buffer	1:1	0:1	0:1	0:1	0:1	0:1	0:1	0:1
Sodium caseinate (mg/mL)	-	-	0.1	1	5	12.5	1	12.5
Lecithin (mM)	-	-	-	-	-	-	0.46	0.46
Glycerol (mg/mL)							2	2
Triolein (mM)	-	-	-	-	-	-	40.25	40.25
pH adjusted to 5 (1 N HCl/NaOH)								

5.2.4. Physicochemical characterisation of the media

All physicochemical properties' measurements were performed in triplicate (n=3) with the results being expressed as mean ± SD.

5.2.4.1. pH

To maintain the pH required, the salt/acid ratio of selected buffer was calculated using the Henderson-Hasselbalch equation (Eq. 5.1) [39],

$$pH = pKa + \log\left(\frac{A^-}{HA}\right) \quad \text{(Eq. 5.1)}$$

where pKa is the acid dissociation constant and A^-, HA are its unprotonated and protonated forms.

A daily calibrated pH meter was used for the measurement of the pH of the media. The pH of milk is approximately 6.7, therefore pH was adjusted to using 1 N HCl in the final medium. Similarly, when NaCas solutions were used in the developed media, pH had to be adjusted. More concentrated NaCas solutions required higher HCl consumption for pH adjustment.

5.2.4.2. Osmolality

The desired osmolality values were achieved by adjustment with NaCl, according to Raoult's law relation (Eq. 5.2) [40],

$$\Delta T_f = i \cdot K_f \cdot m \quad \text{(Eq. 5.2)}$$

where ΔT_f represents the freezing-point depression, i the Van't Hoff factor, accounting for the number of individual ions formed by a compound in solution, K_f the cryoscopic constant (-1.858 K · kg ·mol^{-1} and m the concentration in moles of solute per kilogram of solvent (mol kg^{-1}) or molality of the solution.

Osmolality was measured by determination of the freezing-point of the media using a micro-osmometer. 20 µl of medium was inserted into the instrument's operating cradle and then lowered to the freezing chamber, supercooling the sample. Following a solenoid-induced pulse and subsequent sample freezing, the liberated heat of fusion was related by a microprocessor to the sample's freezing point and osmolality is shown on a digital display [41].

5.2.4.3. Buffer capacity

The concentrations of the un-protonated/protonated forms were selected on the basis of having a solution of pH = 5 and of the desired buffer capacity. The Van Slyke equation (Eq. 5.3) was used to calculate the buffer concentration needed [19],

$$\beta = 2.3C \frac{Ka\,[H_3O]^+}{(Ka+[H_3O]^+)^2} \quad \text{(Eq. 5.3)}$$

where β stands for buffer capacity, C for the total buffer molar concentration (un-protonated and protonated form molar concentrations) and $[H_3O]^+$ for the hydronium ions' molar concentration.

Buffer capacity was measured with dropwise addition of 0.1 N HCl or 0.1 N NaOH, measuring the volume the volume required to change the pH by one unit, under constant stirring. It was calculated according to the following equation (Eq. 5.4) [42];

$$\frac{dB}{dpH} = \frac{\left(\begin{matrix}\text{cc.acid or base added}\\ \text{to cause pH change}\end{matrix}\right)\left(\begin{matrix}\text{normality factor}\\ \text{of acid or base}\end{matrix}\right)}{\left(\begin{matrix}\text{average volume of sample}\\ \text{over range involved}\end{matrix}\right)\left(\begin{matrix}\text{pH change}\\ \text{produced}\end{matrix}\right)} \quad \text{(Eq. 5.4)}$$

5.2.4.4. Surface tension

Surface tension (25 °C) was measured with the Du Nouy ring method [43]. Approximately 10 mL of sample was placed into a glass vessel (Ø 46mm). The ring was submerged below the interface of the sample and help horizontal. After immersion, the ring was pulled up through the surface. The force required to raise the ring from the liquid's meniscus was measured and related to the liquid's surface tension.

5.2.5. Model drug selection and drug solubility studies

Five compounds were selected on the basis of covering a range of lipophilicity values (log P = 0.74-6.2). The compounds studied were: furosemide (log P = 0.74, weak acid), griseofulvin (log P = 2.18, neutral), nifedipine (log P = 2.91, weak base), danazol (log P = 4.2, neutral) and itraconazole (log P = 6.2, weak base).

24 h-solubility values in FeSSGF and Level 2d, 3a and 3b ($FeSSGF_{nm}$) media were determined by using the shake-flask method in FeSSGF [22]. The solubility of the model compounds was determined by weighing excess amounts of the drug into 5 mL Eppendorf tubes, followed by the addition of 4 mL of medium. The samples were left to equilibrate in a shaking water bath at 37 °C for 24 hours. An aliquot of the medium was filtered through a GF/D filter of 2.7 μm pore size to remove undissolved drug particles. To remove the medium's proteins, 1 mL of ACN was added to 0.5 mL of the filtered sample, vortexed at full speed (30 sec). For FeSSGF, the vortexing step was followed by a centrifugation step (15 min, 8000 rpm, 4 °C), while for the other media the sample was filtered immediately through a 0.45 μm RC filter, then diluted 2-200 times (according to the drug solubility) with medium and analysed using HPLC. Drug was quantified against calibration standards in the same media, with the standards undergone the same treatment as the sample. Each measurement was performed in triplicate.

5.2.6. Drug quantification and HPLC analysis

Modified versions of chromatographic methods depicted in the literature were used for drug quantification and are presented in Table 5.2.

Table 5.2. HPLC-UV analytical methods for drug quantification.

Drug	Column	Mobile phase	Flow rate (ml/min)	Temperature (° C)	Inj. Vol. (µl)	λ (nm)
Itraconazole [44]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 80:20	1	35	100	260
Danazol [45]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 75:25	1	25	100	285
Nifedipine [46]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 60:40	1	20	50	238
Griseofulvin [47]	Waters XBridge Shield RP18, 130Å, 150 x 4.6, 3.5 µm	MeOH:H_2O 65:35	0.8	20	50	292
Furosemide [48]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Formic acid 0.1% v/v 60:40	0.8	25	20	233

Filter adsorption studies were performed for all drugs. No significant drug adsorption was observed for both RC (0.45 µm) and GF/D (2.7 µm) filters.

5.2.7. Statistical Analysis

Differences in drug solubility in FeSSGF and Level 2d, 3a and 3b ($FeSSGF_{nm}$) media were evaluated with a two-way Analysis of Variance (ANOVA) (Statgraphics v. XVI, StatPoint Technologies Inc., United States) with a post-hoc Bonferroni test. Effect of different protein content and lipid content on drug solubility was assessed with log P as a covariate in the model. Comparisons where $p < 0.05$ suggested a statistically significant difference.

5.3. Results and Discussion

5.3.1. Physicochemical characterisation of FeSSGF$_{nm}$ levels

The physicochemical properties of FeSSGF$_{nm}$ and intermediate development media (Table 5.1) are presented in Figure 5.1.

The pH value of the medium was mainly controlled by the buffer selected for the medium preparation (acetate buffer pH = 5) and by the milk or casein solution used.

The buffer capacity of the medium is particularly important as it can affect dissolution of ionisable drugs [49]. Buffer capacity was mainly governed by the "blank" buffer (Figure 5.1). A slight increase was observed with increasing the sodium caseinate concentration due to the buffering potential of sodium caseinate solutions, the buffer capacity of which is concentration dependent (Level 2a-2d media) [50]. The buffer capacity of the final medium was 27.12 ± 0.31 mEq/L/ΔpH when the sample was titrated with 0.1 N HCl, which was similar to the value measured for FeSSGF (24.12 ± 0.90 mEq/L/ΔpH). Similar differences between the two media (FeSSGF and FeSSGF$_{nm}$) were also observed with 0.1 N NaOH titration (Figure 5.1).

Osmolality can have an effect on dissolution rate of drugs due to the difference in osmotic pressure created between the inner part of the formulation and the dissolution medium. Big differences in osmotic pressure are associated with higher water penetration and swelling of the formulation [8]. The required osmolality values in FeSSGF and the new medium were adjusted with NaCl, according to the *in vivo* values measured in gastric juice [2]. The mean values of all media were between 390 and 406 mOsm/kg (Figure 5.1).

Surface tension values measured at each level of the novel medium are presented in Figure 1. Surface tension of FeSSGF was measured at 52.3 ± 0.3 mN/m [19], a value significantly higher than the equivalent measured *in vivo* (30-31 mM/m) [2]. It was observed that casein was the main factor responsible for lowering surface tension. The surface tension of the "blank" acetate buffer was 57.8 ± 3 mN/m. The addition of sodium caseinate decreased the medium's surface tension to values of approximately 45 mN/m (media Levels 2a-2d) (Figure 5.1). The surface tension of the medium was not affected by sodium caseinate concentration in the range studied (0.1 to 12.5 mg/mL), possibly because the working concentration range is close or above casein's critical micelle concentration value (0.1 mg/mL) [51]. The addition of polar and non-polar lipids (media Levels 3a, 3b) caused a very slight

decrease by 1-2 mN/m, probably attributed to the small amount of lecithin added (0.46 mM) in Levels 3a, 3b of the medium, which acts as surfactant and has been shown to reduce surface tension in biorelevant media [52].

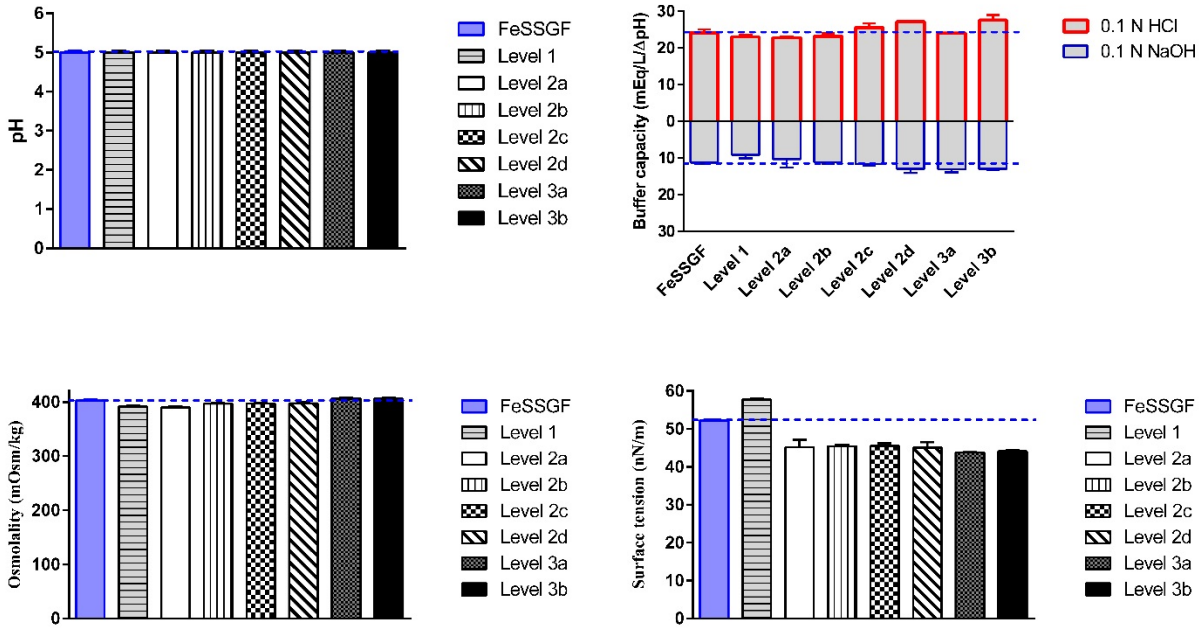

Figure 5.1. Physicochemical properties of FeSSGF and levels of $FeSSGF_{nm}$*.

* Surface tension in FeSSGF as reported in Jantratid et al. [19]

5.3.2. Drug solubility in $FeSSGF_{nm}$ levels

Drug solubility data in FeSSGF and in the last three levels of $FeSSGF_{nm}$ development are presented in Figure 5.2. A log P-dependent solubility was observed for all the media except for level 3b with solubility values ranging from approximately 1 to 8500 μg/mL for the 5 model drugs in the 4 media.

It can be observed that despite the presence of fat in some of the medium levels which facilitates solubilisation of lipophilic compounds, high lipophilicity and solubility in the developed media were still negatively correlated in FeSSGF and level 2d and 3a media (Figure 5.3). Level 2d and level 3a media differ in composition with level 2d medium being a solution of sodium caseinate, not containing any phospholipids or triolein (Table 5.1). Despite the lipids' well-studied positive effect in enhancing the solubilisation of lipophilic drugs *in vivo* and *in vitro* [7], solubility values in these two media were similar (399.4 ± 168.8, 41.51 ±15.24, 17.19 ± 3.87, 4.20 ± 0.41, 1.62 ± 0.37 μg/mL in level 2d and 294.3 ± 18.5, 17.36 ± 0.28, 11.09 ± 5.21, 1.54 ± 0.24, 1.18 ± 0.66 μg/mL in level 3a for furosemide, griseofulvin, nifedipine,

danazol and itraconazole respectively) (Figure 5.2). The similarity in drug solubility values, despite the absence of fat in level 2d medium, may be attributed to the increased amount of casein in medium 3a (12.5 mg/mL vs 1 mg/mL). The physiological role of casein micelles is the distribution of phosphate salts and amino acids to neonates [55], but due to their solubilisation capacity, casein micelles have also shown promising potential as basis of drug delivery systems for poorly soluble drugs like flutamide [56] and celecoxib [57]. Therefore, drugs may be forming complexes with the casein micelles increasing their solubility in the medium. The formation of hydrophobic interactions between non polar regions of drugs and casein micelles, excluding the possibility of electrostatic bond formation has been suggested with the use of fluorescence spectroscopy [53]. Another study for vitamin D, a lipophilic compound, has also reported binding via formation of hydrophobic interactions between the compound of interest and caseinates. [54].

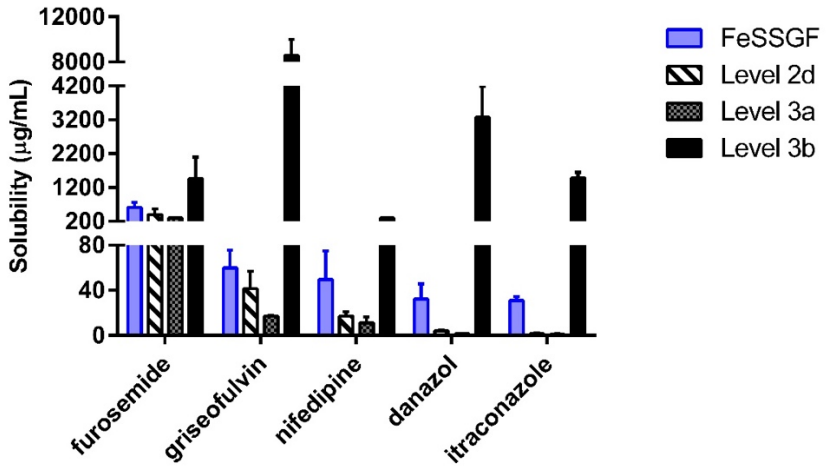

Figure 5.2. 24 h-solubility values in FeSSGF and levels of $FeSSGF_{nm}$ (n=3).

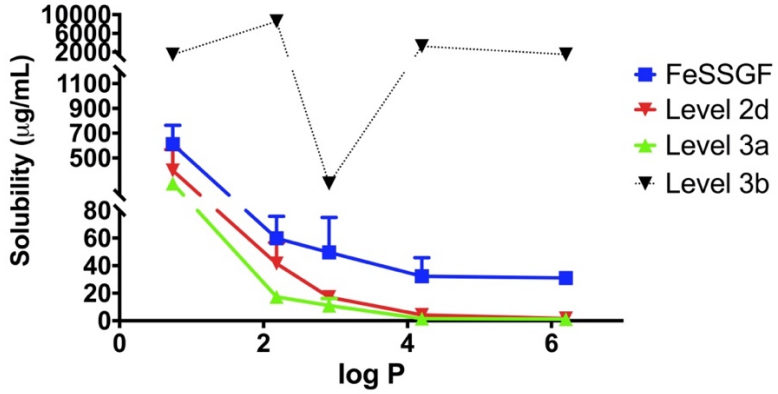

Figure 5.3. 24 h-solubility values in FeSSGF and levels of $FeSSGF_{nm}$ vs drug log P.

$FeSSGF_{nm}$ overpredicted the solubility of all five drugs in FeSSGF, with only solubility values of furosemide and nifedipine being within reasonably comparable limits (1.38 and 4.94 times higher respectively), while solubility of griseofulvin, danazol and itraconazole was 142.3, 100.2 and 46.9 times higher than in FeSSGF (Figure 5.4). The observed differences in the solubility between FeSSGF and the developed medium could be attributed to the difficulty to replicate the sum of the micelles' characteristics formed in FeSSGF. Changes in the size and nature of the surfactant's polar head and length of its non-polar part as well the presence of other solutes in the solution, can alter the aggregation characteristics, shape and size of the micelles formed, affecting thus the medium's solubilisation capacity [58]. Moreover, addition of lipid material has been found to produce aggregates and also increase the variability of micelles regarding their shape and size [52]. In the current study, only one type of polar (E-PC) and non-polar (triolein) lipids was used in order to simulate the lipid membrane and the non-polar core of the medium's fat globules, respectively. The triglyceride core of bovine milk fat globules comprises of triglycerides of more than 400 saturated and unsaturated fatty acids [36]. 29 of the fatty acids have been quantified, with each accounting for more than 1 mg/g of the total fatty acid content [59]. Similarly, substituting the milk fat globule membrane which keeps the lipids emulsified in milk with egg lecithin could be a reason for achieving higher drug solubility values than expected. Except for phosphatidylcholine which represents approximately 35% of the polar lipids of the fat globule, MFGM contains a significant amount of other types of phospholipids (phoshatidylserine, phosphatidylinositol and phoshatidylethanolamine) [37]. It also contains glycolipids, glycoproteins, total and partial glycerides, cholesterol and free fatty acids [37], which for reasons of simplicity were not taken into consideration in the final medium ($FeSSGF_{nm}$) development.

Medium 2d, which did not contain any lipids, underpredicted drug solubility (Figure 5.4) with the developed medium/FeSSGF solubility ratios decreasing with drug lipophilicity. The ratio calculated for furosemide (log P = 0.74) was 0.65 and it progressively decreased, reaching a value of 0.05 in the case of the extremely lipophilic itraconazole (log P = 6.2) (Figure 5.4). Low solubility results of drugs of high partition coefficient values (log P > 4) in the lipid-free medium can be justified by their decreased aqueous solubility and their high affinity in oils [60]. Despite its similar lipid content to FeSSGF and level 3b medium ($FeSSGF_{nm}$), medium 3a demonstrated similar solubility values to the lipid-free medium (Level 2d) (Figure 5.4). A possible reason for the similar solubility values of the lipid-free and level 3a media could be inadequate emulsification of triolein and consequently absence of participation of the

lipid part in drug solubilisation. It is possible that the reduced amount of casein (1 mg/ mL), compared to $FeSSGF_{nm}$ (12.5 mg/ mL) was not adequate for the emulsification of triolein. Level 3a medium demonstrated phase separation within minutes, with the shaking conditions in solubility studies not sufficing for the homogeneous distribution. Increased casein content acted as a surfactant [61], helping the non-polar lipids suspend in the medium. Protein and lipid contents of medium were deemed to affect drug solubility significantly ($p < 0.05$), showing increased solubility values for the higher levels of lipid and protein contents. Drug lipophilicity (log P) did not affect significantly the above dependence on lipid and protein contents ($p = 0.34$).

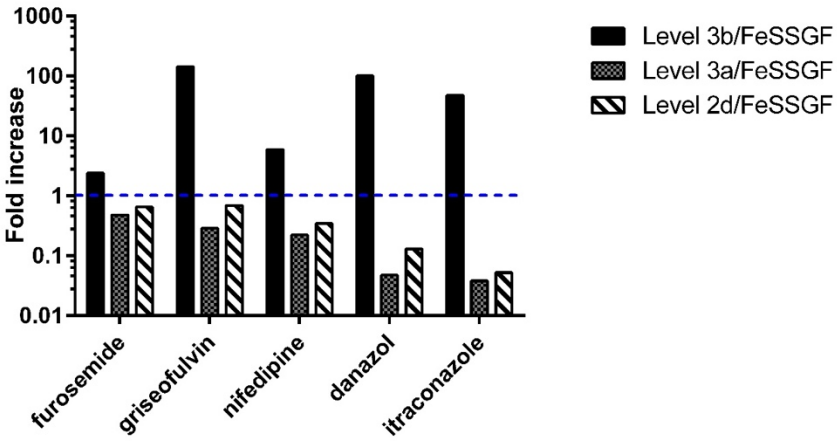

Figure 5.4. Difference in solubility obtained in the $FeSSGF_{nm}$ levels compared to FeSSGF.

5.4. Conclusion

In the last years, several media simulating the fed state gastric environment have been developed and used in biorelevant solubility and dissolution studies, with the role of fat content as a factor affecting drug pharmacokinetics after meal administration being extensively studied and included *in vitro* medium development. In the current study, a medium of physicochemical properties similar to $FeSSGF_{middle}$ was developed, which required a simpler extraction treatment, saving time and effort in drug analysis. $FeSSGF_{nm}$ was developed on the basis of simulating drug solubilisation in the casein micelles and lipid components of milk contained in $FeSSGF_{middle}$. In the range of drugs selected, the new medium overestimated drug solubility, an effect which was more profound for lipophilic drugs. Taking fat content into consideration for drugs of moderate to high lipophilicity though was essential, as pointed by the lower solubility values observed when triolein was not a part of the medium's composition. Both casein and lipids were found to have a positive effect on drug solubility. Future work should

focus on the deeper understanding of the drug solubilisation mechanism in milk's casein micelles and lipid components. Additionally, simulation of the lipid core and tri-layer membrane of milk fat globules could be proven valuable towards the development of a medium demonstrating both biorelevance and simplicity in analysis.

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Conclusions and Future Directions

Conclusions

The assessment of food effect in drugs' pharmacokinetic behaviour has been extensively investigated over the last decades. Both EU and US regulatory agencies have mandated food effect studies in orally administered medicinal products for both immediate and modified-release oral formulations. Versions of a high-fat standardised breakfast are proposed by both EMA and FDA as reference meals administered in *in vivo* studies. The need for simulation of the above meals in the gastric compartment, with the aim to predict food effect *in vitro,* was addressed by use/development of biorelevant media which would take into consideration the composition and physicochemical characteristics of the GI tract. The effect of meal fat content has been characterised as of great importance for the prediction of the drugs' pharmacokinetic behaviour and has been included in the majority of the media developed so fat. Despite the successful employment of such media, two main drawbacks associated with the media's heterogeneous nature arose; complexity in sample treatment prior to its analysis and lack of a unified analytical guideline deriving from the drug's physiochemical characteristics and interaction with medium components. The current project gave an insight on three main aspects considered important towards the understanding of drug's distribution in the simulated gastric media (i. API-medium interactions, ii. excipients-medium interactions and iii. API-fat interactions). The ultimate goal of the project was to use the above observations so as to set the basis for a predictive protocol which uses drug/formulation properties as input variables towards the selection of an optimal analytical protocol in fed state gastric biorelevant media.

The current status and recent developments towards the *in vitro* simulation of the fed gastric environment were presented in Chapter 1. The effect of the gastric environment on the drug's pharmacokinetic behaviour is governed by a number of parameters, the most important of which are: i. API's physicochemical properties, ii. formulation characteristics, iii. type of meal administered, iv. presence of lipolysis enzymes and v. *in vivo* hydrodynamics. Most of the different media currently used were developed on the basis of reflecting the gastric properties (pH, osmolality, surface tension, buffer capacity) measured after administration of solid or liquid meals. So far, the use of lipolysis enzymes in gastric media has been relatively limited in such media. A number of techniques has been used as pre-treatment steps for the analysis of such media such as protein precipitation, solid phase extraction and liquid-liquid

extraction. In all relevant studies cited in Chapter 1, a pattern was observed; lack of a commonly accepted medium for gastric fed-state studies *in vitro*, lack of reasonably simple and time efficient extraction techniques and a case-by-case approach for the analysis of each drug according only to its own unique physicochemical characteristics.

Protein precipitation and solid phase extraction are two sample clean-up techniques very commonly used prior to analysis of compounds dissolved in biological fluids or heterogeneous media, for which simple filtration cannot provide a "clean" sample. Protein precipitation was proven to be effective for the extraction of compounds of a wide range of lipophilicity, with the selection of appropriate reagents added to an adequate amount (at least 1:2 medium:reagent ratio). A dependence between the amount of drug recovered and specific physicochemical characteristics of the compounds of interest (lipophilicity, ionisation, aqueous solubility, protein affinity) was demonstrated, which can potentially serve as the basis of selection of a compound's extraction conditions based on the above properties. The equivalent predictive potential of solid phase extraction is significantly more limited. Despite demonstrating an improved efficiency for compounds of intermediate lipophilicity, the most commonly used C_{18} cartridges could discriminate among drugs of different physicochemical characteristics. While HLB cartridges can potentially be used for drugs of higher lipophilicity which are adequately soluble in water (according to the PLS regression analysis), the higher cost/time consumed and the lower amount of drug recovered make solid phase extraction a secondary choice in biorelevant media analysis.

Protein precipitation was proven effective for drugs of a wide range of lipophilicity and ionisation, but the presence of certain excipients can affect the extraction potential of the protocols for the analysis of active substances developed in Chapter 2. Certain excipients (magnesium stearate, HPMC) decreased the amount of the extracted drug when mixed with APIs, an effect more profound for lipophilic drugs (according to the MLR analysis). Interactions between excipients with the APIs and medium components affect the efficiency of the protocols developed; the above extent though was much smaller in solid dosage forms and was only observed in physical mixtures. Therefore, the effect of excipients in drug analysis should be mainly investigated in cases of drugs administered in the form of powders or liquid formulations.

Drug partition behaviour to the lipid portion of the gastric content may be a potential indicator of changes in drugs' pharmacokinetic parameters when administered with high fat

meals. The work in Chapter 4 revealed a possible correlation between the drugs' rate of partition to fat and lipophilicity, a behaviour which is also controlled by other physicochemical properties. Regression analysis showed that high drug lipophilicity, low molecular weight and high aqueous solubility in the working pH, are the parameters to which the rate of partition is most positively correlated. Moreover, drugs of negative food effect experience low partition rates, which is in accordance to the initial hypothesis associating the rate of drug distribution to fat with food effect observed *in vivo*. The inclusion of lipolytic enzymes in biorelevant concentrations does not appear to change the partition behaviour observed.

The need for a gastric fed biorelevant medium, which would be easier to analyse than the lipid emulsions and milk-based media currently used, has not been yet fully met. In this thesis, a stepwise approach produced a medium with similar physicochemical properties to FeSSGF, requiring a simpler sample clean-up process. The effect of the casein micelles and lipid content on drug solubilisation were clearly observed, but the simulation of the emulsified fat globules in milk was revealed to be more complicated than expected as drug solubility values can change significantly by small alterations in content of polar/non-polar lipids and casein.

Future Directions

The research portrayed within the project was an attempt to build a predictive model on the analysis of a wide range of drugs in fed state media and assess relationships among drug physicochemical properties, analytical aspects and food effect observed *in vivo*. With the focus of the current study on gastric fed state, it is evident that for the development of a generally applicable predictive model, other parameters responsible for a potential food effect must be incorporated. The roles of gastric residence and *in vivo* hydrodynamics should also be considered in the final model in regard to the simulation of the gastric compartment, while variations of the type of meal administered may need to be considered too. The inclusion of an equivalent model built for the fasted intestinal state, to be used in conjunction with the gastric, may report different findings and lead to clearer correlations with food effect.

In terms of drug analysis in fed gastric media, expansion of the work performed in the analysis of milk-based media should be expanded to lipid emulsions, which are widely used in *in vitro* simulation of the fed stomach content. The development of a roadmap for selection of optimum working conditions in both types of media can be a determinant step towards an automated selection of analytical processes, avoiding the case-by-case approach currently used.

With regards to excipients, the matrix-excipient interaction hypothesis stated in chapter 3 should be confirmed experimentally, using a microscopy technique. Moreover, evaluation of the effect of the manufacturing process (in terms on how the API and excipients are mixed) on the extraction process could be beneficial; evaluation of each excipient type separately or in combination with others can give more definitive answers on drug-excipient interactions in both solid and liquid oral dosage forms.

To evaluate the full extent of the effect of drug-fat interactions to possible food effect, research is still required for the full understanding of both partition and release processes to and from gastric content's lipid portion if the fed state. As mentioned in Chapter 4, food effect on bioavailability depends on a number of complex mechanisms and therefore, light must be shed to several aspects of drug-lipid interactions in order to develop a food effect-predictive assay. It is possible that the different types of dietary fats and oils demonstrate a different behaviour in regard to their interaction with fat. Therefore, future work in developing *in vitro* setups with lipids more representative to the high-fat standardised meals administered in fed state BE studies may be able to elucidate lipid induced-food effect mechanisms due to better biorelevance. Further studies should also investigate parameters such as the role of the meal composition, compound's surface activity and excipients present in the formulations affecting the rate of drug partition to the lipid portion of the gastric content. Future work should investigate the rate of drug "release" from the lipid part of the gastric fed content to the intestinal environment. A drug release setup using FeSSIF (Fed State Simulated Intestinal Fluid), a biorelevant medium representative of the fed intestinal environment, as dissolution medium is proposed as a first step for future consideration. The combination of the above partition and release processes in a single experimental setup and the assessment of their cumulative effect can potentially lead to a better understanding of the mechanisms of fat-induced food effect in the GI tract. Knowledge of these interactions must be incorporated to the development of new media, more biorelevant and preferably simple in analysis. The development of fed gastric media requiring simpler sample clean-up prior to drug analysis, as opposed to the existing milk-based ones or lipid emulsions, will help pharmaceutical scientists overcome analytical issues related to sample treatment and save significant time. As demonstrated in the last chapter, the biorelevant simulation of the fat globules is a prerequisite for the design of the desired alternative medium similar to FeSSGF (or in the development of novel media mimicking the *in vivo* conditions). Finally, the role of gastric lipolysis, the effect

of which has only relatively recently been taken into consideration in biorelevant dissolution, should be further explored.

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